Microalgae in Sewage Treatment

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Abstract

Anaerobic digestion is a common method of treating sewage sludge; however the anaerobic digestate liquor (ADL) contains high nutrient levels that have to be treated. The aim of this study was to determine whether microalgae could be used to strip nitrogen (N) and phosphorous (P) from ADL produced at Bran Sands sewage treatment works (STW); whether the ADL contained toxins and/or missing nutrients; and identify the spatial and temporal parameters of a potential bioremediation system. ADL was collected from Bran Sands STW and *Scenedesmus obliquus* was grown on various dilutions of ADL at laboratory scale. It was found that microalgae grown on the ADL was dually inhibited by high ammonia (NH_3) concentrations and limited trace element availability. Trace element limitation decreased the microalgae biomass productivity to 1/3 of cultures with trace element supplementation. It was also found that NH_3 concentrations > 17-23 mg/l NH_3-N completely inhibited growth. A long time lag observed in 10 % ADL solutions was found to be due to the pH decreasing in ADL flasks over time (from 9.30 to 8.60), leading to a decrease in NH_3 concentration until the toxicity threshold was crossed (approximately 20 mg/l NH_3-N at pH 8.80), after which exponential growth occurred. Using 17.62 mg/l NH_3-N as an inhibition threshold, it was calculated that the highest concentration of total ammonia nitrogen TAN that could be remediated at pH 7.0 was 400 mg/l TAN (a 3.75 × dilution of neat ADL). Based on observed growth and nutrient uptake rates, it was further calculated that a microalgae remediation system could strip 400 mg/l TAN from the ADL within 5.66 days. If growth conditions were optimized and the growth rate could be increased to > 1.0 d^{-1} (reported by Ho et al. (2010)) the remediation time could be reduced to 3.31 days (although this does not consider other growth-limiting factors such as light inhibition). Due to fluctuating N:P ratios it was not possible to calculate predicted P uptake.

Declaration

I confirm that no part of the material presented in this thesis has previously been submitted by me or any other person for a degree in this or any other university. In all cases material from the work of others has been acknowledged.

Copyright

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List of Abbreviations

Abbreviation	Meaning	Page of first mention
10%-ad	Previously grown on 10 % ADL	p18
AD	Anaerobic digestate	p2
ADL	Anaerobic digestate liquor	p2
BBM	Bold's Basal Medium	p10
CCAP	Culture Collection of Algae and Protozoa	p10
DI	Deionised	p9
GM	Growth medium	p26
GrM-ad	Previously grown on growth medium	p18
IC	Ion chromatography	p9
LB	Lysogeny broth	p14
OD	Optical density	p15
NWL	Northumbrian Water Limited	p1
S. obliquus	*Scenedesmus obliquus*	p10
SAG	Sammlung von Algenkulturen	p10
SBBS	School of Biological and Biomedical Sciences	p7
STW	Sewage treatment works	p2
TAN	Total Ammonia Nitrogen	p39
TEM	Transmission Electron Microscopy	p15
TN	Total nitrogen	p6
WWTP	Wastewater treatment plant	p1

Nitrogen and phosphorus nomenclature

The terms "ammonium" and "ammonia" are referred to a great deal within this thesis. The term ammonium is used to describe the ionized species (NH_4^+) and the term ammonia (or free ammonia) refers to the unionized species (NH_3). The distribution of the two species is determined by the pH and temperature conditions. However the molecular weights of these two species (NH_3 and NH_4^+) are different. Thus the molecular weight of the nitrogen within these species is referred to as ammonia-nitrogen (NH_3-N) and ammonium-nitrogen (NH_4-N), to make the nitrogen concentrations comparable. Similarly PO_4-P refers to the concentration of phosphorus atoms within phosphate ions in a solution, not the concentration of phosphate molecules. As concentration is stated in units of mass per unit volume in this study, this nomenclature becomes important. Total ammonia nitrogen (TAN) refers to the concentration of both NH_3-N and NH_4-N. Total nitrogen (TN) refers to the concentration of nitrogen present in all the nitrogen species measured in this thesis (ammonium-N, nitrite-N and nitrate-N).

1. Introduction

Every day in the UK, more than 11 billion litres of wastewater are collected by 624 200 km of sewers (DEFRA, 2012). This wastewater comes from homes, municipal, commercial and industrial premises and rainwater runoff, and although most of it is water (typically < 0.1 % is solid) (DEFRA, 2012), inadequate treatment of this wastewater could have extremely damaging effects on the environment and public health. Therefore it is in everybody's best interest for wastewater treatment plants (WWTP), such as those owned by Northumbrian Water Limited (NWL) to be as efficient as possible.

1.1 Wastewater Treatment Process

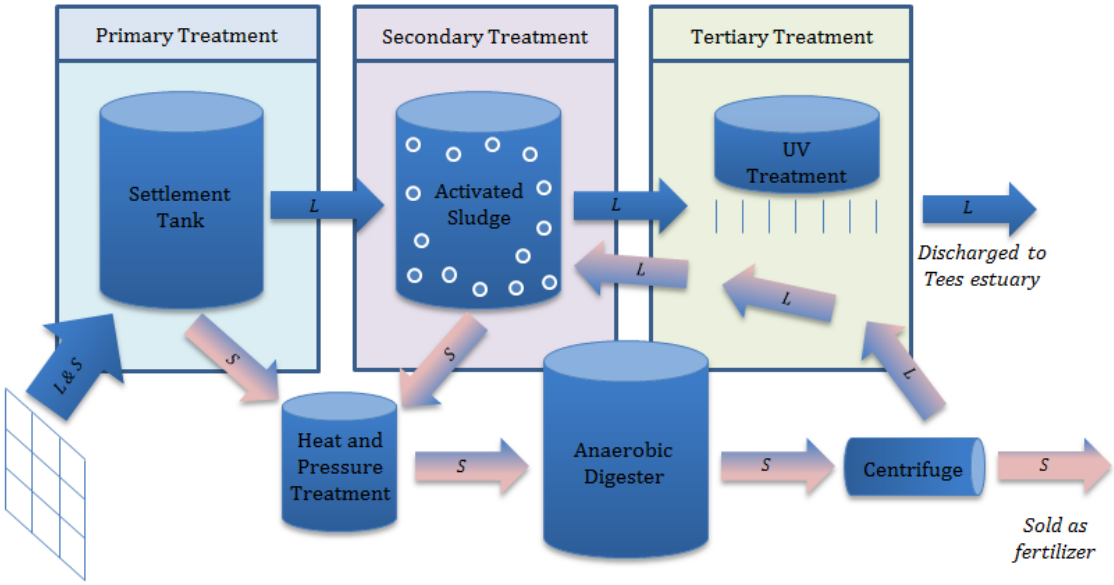

Figure 1: Flow chart showing the treatment process at Bran Sands Sewage Treatment Works. L = liquid, S = sludge.

The current water treatment set up at the NWL owned Bran Sands site involves preliminary, primary, secondary and tertiary treatment. The preliminary stage is a coarse grid that removes large items. The primary treatment stage is a settlement tank where suspended solids sink to the bottom and can be removed as primary sludge. The secondary treatment stage consists of an aerated tank containing "activated sludge", i.e. a host of microorganisms which break down organic matter still present in the water. The tertiary stage uses ultraviolet light treatment to kill pathogens and meet the requirements of the Bathing Water Directive (EU, 2006). The final treated water is then released into the river network and coastal waters. The sludge from the primary and secondary stages is heated to 165 °C and held under 6 Bar pressure for 45 minutes to kill pathogens (NWL, 2011). This sterile sludge is then added to an anaerobic digester for 18 days to decrease volume and recover energy. The process is shown in Figure 1.

In the last decade, the use of anaerobic digestion to treat sewage sludge has become increasingly commonplace and around two thirds of all UK sewage sludge is now treated in this fashion (Environment Agency, 2013). Anaerobic digestion is a mature industrial process that decomposes

organic matter by using bacterial populations to convert the biomaterial to methane in controlled anoxic conditions (DEFRA, 2011). NWL operates one of the largest anaerobic digestion systems in the UK at Bran Sands sewage treatment works (STW) on Teesside. Anaerobic digestion already delivers significant cost savings over previous sludge drying treatments (NWL, no date), as well as beneficial waste heat, biogas and reductions in sludge volume (Caldwell, 2009). The biogas is a mixture comprised mainly of carbon dioxide and methane, which can be upgraded for injection to the gas grid through being enriched with higher carbon gases and then used to generate electricity or compressed to form a liquid fuel (Bjornsson et al., 2013). The biogas currently runs a gas engine that delivers local electricity (Caldwell, 2009), with biomethane delivery into the grid also being developed as a longer-term option (Andrew Moore and Chris Greenwell, NWL and Durham University, personal correspondence, 2013).

At Bran Sands STW, biomethane produced during the anaerobic digestion process is constantly harvested over the 18 days the sludge is in the digester. After 18 days the remaining anaerobic digestate (AD) is removed from the digester and centrifuged. The sludge fraction of the digestate, which contains high N and P levels, can be used to provide a high quality fertilizer for crop plants (DEFRA, 2011). The liquid portion is returned to the secondary treatment stage of the plant (Figure 1). However, this liquid fraction of the AD has very high nutrient concentrations and although the return AD liquor (ADL) typically only contributes ≈ 2 % of the total influent, it can contribute up to 25 % of the total inlet nitrogen load (Janus and van der Roest, 1997). Therefore, if this stage was treated to decrease nitrate, ammonium and phosphate concentrations, the nutrient emissions of the entire plant to the outflow could potentially be lowered.

1.2 Eutrophication

Ensuring the nutrient emissions of WWTPs remain low is important for environmental and legal reasons. Many UK rivers and estuaries are adversely affected by such wastewater pollution (Neal et al., 2010). High nitrate and phosphate concentrations, mainly derived from over application of fertilizers to arable land, often drive coastal and estuarine eutrophication (Withers and Lord, 2002, Mainstone and Parr, 2002). Farming contributes approximately 60 % of nitrates and 25 % of phosphates entering UK waters (DEFRA, 2010). However, point sources such as sewage outlets are still a significant contributor to eutrophication in the UK (Jarvie et al., 2006). The ecological impacts of eutrophication can include reduction of biodiversity, replacement of dominant species, increased turbidity and increased water toxicity (Cai et al., 2013a). For this reason legislation such as the EU Water Framework Directive (EU, 2000) has been passed with an aim to reduce nutrient pollution in rivers, lakes and coastal areas. This puts pressure on the water industry to reduce N and P discharges into waterways and coastal areas.

1.3 Bioremediation

There are a number of methods used to remove nutrients from wastewater, including precipitation of ammonium and orthophosphate to struvite, ammonia stripping and the biological removal of

nitrogen (nitrification/denitrification, nitritation/denitritation and deammonification) (Jardin et al., 2006). One method that has received a great deal of attention over the past few years is microalgae bioremediation.

Bioremediation is the use of microorganisms to consume environmental pollutants and thus remediate a polluted waste stream or site. NWL is interested in using bioremediation to reduce the cost of its activated sludge stage, which currently treats all of its ADL. This will be achieved by growing microalgae upon the ADL to strip out excess ammonium and phosphate. Additionally NWL is interested in the possibility of recovering a valuable by-product from the produced microalgae biomass, as either chemicals or biofuels. Microalgae treatment could also have the positive side effect of reducing the amount of nitrate/ammonium and phosphate delivered into the Tees estuary as aqueous discharge from the plant.

1.4 Microalgae overview

Microalgae are mainly photosynthetic oxygen-producing microorganisms that contain chlorophyll *a*. Microalgae can be autotrophic, utilizing atmospheric CO_2 as their primary carbon source; mixotrophic (facultatively using a previously fixed source of carbon as well as CO_2); or heterotrophic (exclusively using organic sources of carbon – these microalgae do not produce O_2 as they do not photosynthesize). Microalgae can be single or associated cells and can be found in and on oceans, rivers, lakes, soils and rocks and in a variety of pH and salinity conditions. Therefore the term "microalgae" is one that applies to tens of thousands of species belonging to several kingdoms (Leite et al., 2013).

One of the most commonly studied phyla in relation to wastewater remediation is the chlorophytes (green algae). Chlorophyta is one of the largest phyla of microalgae with an extensive range of species distributed over a large geographic area. Two genera that are commonly used to remove nutrients from wastewater in scientific studies are *Chlorella* sp. and *Scenedesmus* sp. (Cai et al., 2013a). The species used in this study is *Scenedesmus obliquus.* The choice of species is discussed in Section 2.4.

Microalgae uptake inorganic nitrogen (in various forms) and phosphorus in the form of phosphate from their surrounding environment for the purposes of cell building (Cai et al., 2013a). Thus microalgae can be used to decrease nitrate, ammonium and phosphate concentrations in wastewaters. A great deal of research has focused on microalgae due to its suggested potential as a third generation biofuel. Microalgae have a fast growth rate; most species double once per day but some can double every few hours (Alam et al., 2012). They can also have high oil contents (20-50 % on a dry weight basis) (Williams and Laurens, 2010), however this tends to be in N-limiting conditions when they are stressed and thus growth rate is reduced (Kenny and Flynn, 2015). Microalgae are suitable feedstock for the production of biodiesel (Chisti, 2007), bioethanol (John et al., 2011) and biogas (Frigon et al., 2013). Depending on the species, it is also possible to extract high-value chemical compounds from microalgae biomass, such as pigments, antioxidants, β-carotenes, polysaccharides, triglycerides, fatty acids and vitamins (Mata et al., 2010). Additionally, algae

aquaculture does not necessarily compete for arable land (as it can be grown on marginal land), or fresh water (if wastewater is used as a growth medium) (Greenwell et al., 2010).

As an anaerobic digestion unit is already present on site at Bran Sands, growing microalgae on ADL and then using the biomass as feedstock for the anaerobic digester could be a sustainable, environmentally friendly method of remediating Bran Sand's wastewater and obtaining energy to run the plant (Frigon et al., 2013). Use of carbon-neutral energy will also help Northumbrian Water meet the requirements of the Climate Change Act (UK Parliament, 2008).

1.5 Nutrients required for microalgae culture

The three key nutrients needed for microalgae growth to occur are carbon, nitrogen and phosphorus. When microalgae are grown autotrophically they use utilize dissolved CO_2 as the substrate for RUBISCO in the photosynthetic pathway. However not all microalgae species are obligatory photoautrophs, so species can assimilate carbon heterotrophically or mixotrophically and can therefore utilize other carbon sources such as dissolved organic or dissolved inorganic carbon (Cai et al., 2013a). Organic cellular nitrogen is derived from inorganic sources including nitrate (NO_3^-), nitrite (NO_2^-), nitric acid (HNO_3), ammonium (NH_4^+), ammonia (NH_3) and certain cyanobacteria are able to uptake nitrogen gas (N_2) if no other forms of dissolved inorganic nitrogen are available. Studies have shown that algae generally prefer ammonium, a reduced form of N, to nitrate, so nitrate consumption does not tend to occur until ammonium concentrations have been almost entirely depleted (Maestrini et al., 1986). This means that wastewaters with high levels of ammonium can effectively cultivate microalgae. However excessive levels of ammonium can have an inhibitory effect (Morris and Syrett, 1963). It should be noted that environmental conditions can also remove ammonium from solution; ammonia stripping can occur due to high temperatures and high pH (García et al., 2000). Organic phosphorus is derived from inorganic phosphates ($H_2PO_4^-$ or HPO_4^{2-}). Again, cell uptake is not the only way phosphorus can be removed from wastewaters. High pH and dissolved oxygen concentrations can cause phosphorus to precipitate and be removed (Alcántara et al., 2013).

AD (and thus ADL) typically has high nitrogen and phosphorus concentrations (1.20-9.10 mg N/g fresh digestate and 0.4-2.6 mg P/g fresh digestate) and high ammonium: total nitrogen ratios (44-81 %) (Moeller and Mueller, 2012). The large quantities of ammonium are generated by the degradation of proteins (Smith K A et al., 2007). ADL has 25 % more accessible NH4-N than untreated liquid manure (Smith K A et al., 2007) and the C:N ratio of the organic materials in AD is typically 20-30 (Monnet, 2003). AD also typically has a high pH due to formation of ammonium carbonate ($(NH_4)_2CO_3$) and the removal of CO_2 (Moeller and Mueller, 2012).

The ADL in this study has high nutrient concentrations (822 mg/l NH_4-N and 111 mg/l PO_4-P) potentially make ADL an ideal substrate for microalgae growth.

Nitrogen and phosphorus are the two nutrients most commonly associated with eutrophication, as they are typically the nutrients limiting growth in most scenarios and once provided growth of microalgae may be rapid (Anderson et al., 2002). Other micronutrients can be growth limiting if absent, such as magnesium (Bjornsson et al., 2013), silicon (Brzezinski et al., 1990) and iron (Petrou et al., 2011). On the other hand many micronutrients can be toxic to algal species at high levels (Seidl et al., 1998).

Microalgae productivity can potentially be improved by having ideal nutrient ratios within the growth medium (Bjornsson et al., 2013) however this is a complicated issue. Microalgae growth can be limited by nutrient deficiency and thus a growth medium is most efficient if the microalgae are able to uptake all the N and P before growth inhibition occurs due to dual-limitation of N and P. In marine algae the molar elemental N:P ratio typically corresponds to the Redfield ratio (16:1) (Redfield, 1958). The N:P ratio is usually higher than this in fresh waters, but tends be lower, around 4-5:1 in wastewaters (Cai et al., 2013a). However microalgae are limited by internal nutrient reserves, not external nutrient concentrations and they also alter the rate at which they uptake nutrients depending on the external availability (Flynn, 2010). For these reasons C:N:P ratios can be highly variable and ensuring that the necessary total concentrations of N and P are available is more important than manipulating the ratio at which they are present.

Another way to increase productivity is to increase the dissolved CO_2 concentration present in the culture media (Lin et al., 2012). This can be done through the use of CO_2 to adjust pH or by bubbling flue gas through the culture to reduce the greenhouse emissions of local industry (Jiang et al., 2013, Leite et al., 2013). Other environmental factors that can affect nutrient uptake and productivity are pH, photon flux density, temperature and inoculation density (Cai et al., 2013a). Additionally it is vital to ensure that these factors are balanced. For example a high initial inoculation density should increase biomass production, however if it is too high it can have a negative impact, as Kenny and Flynn (2015) found that after a certain critical optical depth light limitation becomes more important than nutrient limitation.

1.6 Microalgae growth on wastewater

1.6.1 Microalgae growth on municipal wastewater at high latitudes

Many recent studies have focused on microalgae growth in wastewater. Arbib et al. (2013b) successfully cultivated *Scenedesmus obliquus* outdoors on secondarily pre-treated municipal wastewater at the Arcos de la Frontera (36◦44'56.56''N, 5◦47'37.12''W, Spain). Boelee et al. (2013) treated municipal wastewater in Leeuwarden, Netherlands, using a biofilm photobioreactor. Outdoor high rate algae ponds (HRAPs) have also been used to treat municipal wastewater in Christchurch, New Zealand (Craggs et al., 2012) and Spain (Passos et al., 2013).

1.6.2 Microalgae growth on anaerobic digestate liquor

Some studies have specifically focused on cultivating microalgae on ADL. ADL usually needs to be treated before microalgae can be cultivated upon them due to the high levels of ammonia and light

penetration. The high ammonia concentrations in raw AD wastewater can lead to ammonia toxicity and growth inhibition if microalgae are cultivated upon it (Cho et al., 2013). Additionally the effluent is usually black due to the presence organic matter (Shi et al., 2013) so needs pretreatment to allow sufficient light penetration for photosynthesis to occur (Sahu et al., 2013). Sahu et al. (2013) investigated methods of pretreatment to achieve light penetration without dilution, such as oxidation, particle removal and flocculation as stand-alone and combination processes. However dilution is the most common method of pretreatment of AD effluent as it is simple, cheap, dilutes toxins and the only real downside is the greater quantity of wastewater needing to be remediated.

Cai et al. (2013b) grew *Nannochloropsis salina* on municipal ADL and achieved nitrogen and phosphorus removal rates of 35.3 mg l^{-1} d^{-1} and 3.8 mg l^{-1} d^{-1} respectively. These results were surprising as the N/P ratio of the effluent was 7, which was lower than the atomic ratio of *Nannochloropsis salina* at 16. This lead the authors to believe that *Nannochloropsis salina* may be able to uptake excess phosphorus. Cho et al. (2013) isolated *Chlorella* sp ADE5 from municipal ADL in Busan, Korea. *Chlorella* sp. ADE5 achieved almost 100 % removal of the 250 mg l^{-1} total nitrogen (TN) and 17 mg l^{-1} total phosphorous (TP) from the diluted ADL over 120 hours. It also had a higher biomass production on ADL (3.01 g-dry weight cell l^{-1}) than synthetic growth medium (1.75 g-dry cell weight l^{-1}). Cho and colleagues speculated that either the strain was specifically adapted to grow on wastewater, or that there may be more growth-promoting bacteria present in the wastewater. Bjornsson et al. (2013) examined the possibility of growing microalgae on ADL from the co-digestion of swine manure and algal biomass. *Scenedesmus* sp AMDD removed 100 % of the 1.65 ± 0.03 x 10^{-3} mol l^{-1} NH_3-N (23.1 mg/l NH_3-N) and 99.8 % of the 2.17 x 10^{-4} mol l^{-1} PO_4-P (6.72 mg/l PO_4-P) removal from ADL diluted with lake water.

The aim of this study was to determine whether the ADL at NWL is a suitable growth substrate for microalgae, as well as to investigate whether the microalgae could be used to strip N and P from the ADL and how efficiently this process could be done. The first key objective of the study was to determine whether microalgae could grow on the ADL. The literature showed that the ADL would likely be inhibitory to microalgae growth above certain concentrations. If growth was inhibited, the next objective was to identify whether the ADL contained toxins and/or was missing essential nutrients. The third objective was to identify the best way to counteract these inhibitory effects. The final objective was to use the recorded growth rate and nutrient uptake data to quantify the spatial and temporal parameters of a microalgae bioremediation system treating ADL from Bran Sands STW.

2. Methods

2.1 Sample collection and storage

At the Bran Sands STW, the AD is separated into two fractions using a centrifuge. The sludge fraction is removed by a screw lift, stored in a covered open-air structure and then sold as fertilizer. The liquid portion (used in the experiments in this thesis) is piped back to the secondary stage of the plant. There is a tap on this pipe, from which the samples were collected into new, sterile plastic containers. This process is shown in Figure 2. The samples were then driven to Durham University (approximately a one hour drive) where they were stored at 4 °C in a cold room, at the School of Biological and Biomedical Sciences (SBBS). Three collections, and hence batches of ADL were used in this body of work. There were denoted as: AD1, AD2901 and AD1703.

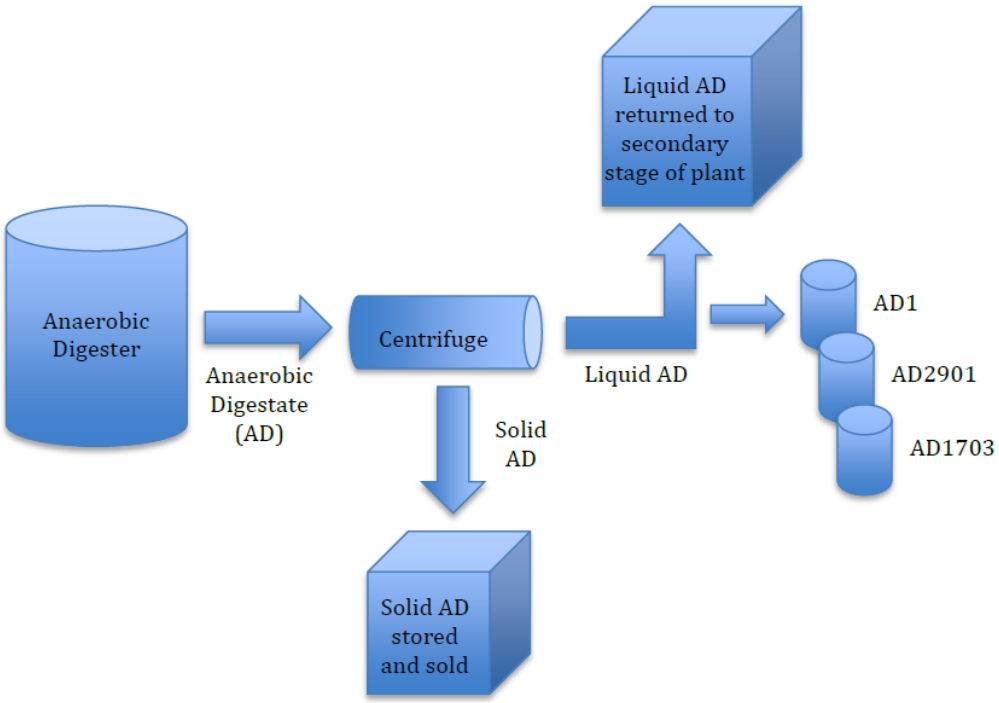

Figure 2: Flow chart showing origin of samples used in this thesis. AD1, AD2901 and AD1703 refer to batches of ADL sampled on 21/11/2013, 29/01/2014 and 17/03/2014 respectively.

2.1.1 Samples AD1, AD2901 and AD1703

The treatment of each of the three batches of digestate used in these experiments is shown Figure 3, Figure 4 and Figure 5. Between 3 – 5 l of ADL was collected each time, in new, clean plastic bottles. The ADL was transported between Bran Sands STW and the SBBS by car. ADL samples were always collected, transported and stored within 6 hours. All AD1 and AD2901AD liquor samples were stored at 4 °C in a cold room in SBBS (storage durations shown on Figure 3 and Figure 4). Half of the autoclaved AD1703 samples were stored in the cold room and half were stored in a -12 °C freezer (Figure 5). Samples AD1 and AD1703 were autoclaved at 121 °C and 2 bar of pressure for 21 minutes, while AD2901 was autoclaved in error at the same pressure and temperature for 50 minutes. However the extra time spent in the autoclave did not appear to affect the key nutrient concentrations (see Figure 8 and Figure 9).

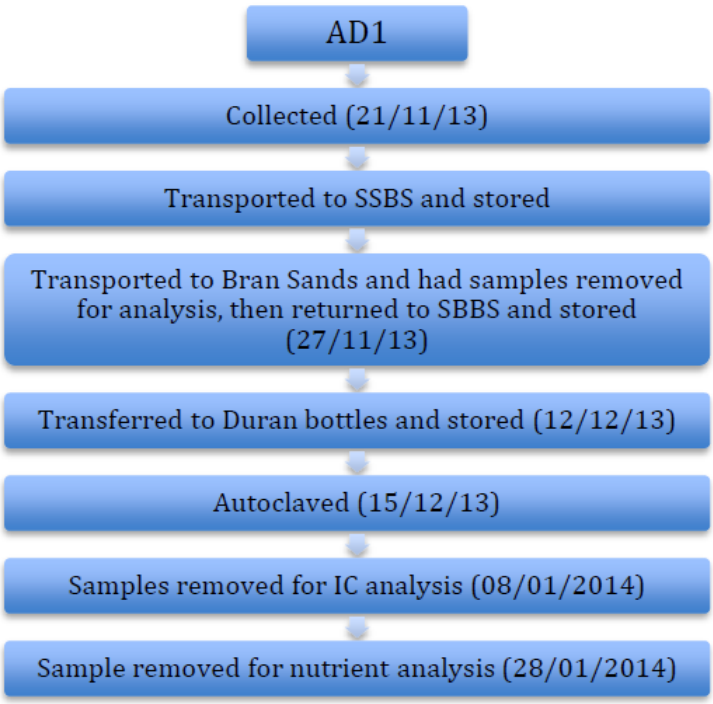

Figure 3: AD1 collection, treatment and usage summary. AD1 was the first ADL sample collected from Bran Sands STW in this study and was not used for many experiments due to the long time period between collection and autoclaving.

No growth experiments were carried out with AD1, as it was considered that the long gap between sampling (21/11/2013) and autoclaving (15/12/2013) might have allowed microbes to change the composition of the ADL. This batch of ADL was used in the initial stages of this study, to investigate the spectral signature of the ADL (Appendix A1iii and A1vi); test how autoclaving ADL affected its nutrient concentrations (Appendix A2); and to check that the methods of nutrient analysis used by NWL (see section 2.2) and those used in this investigation (Section 2.3) were consistent (Appendix A2).

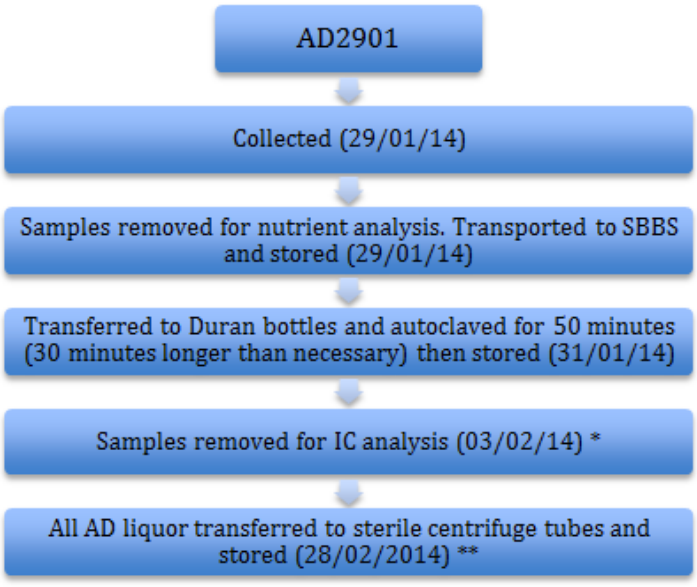

Figure 4: AD2901 collection, treatment and usage summary. * = Process carried out in sterile conditions. ** = The ADL was transferred in a laminar flow hood, to sterile centrifuge tubes.

AD2901 was used in Experiment 1 and Experiment 2 (see Section 2.14). AD2901 was an unusually clear sample (very few suspended solids and high visual transparency). The one month gap between collection and autoclaving was due to the fact I collected the sample under the supervision of staff members, however

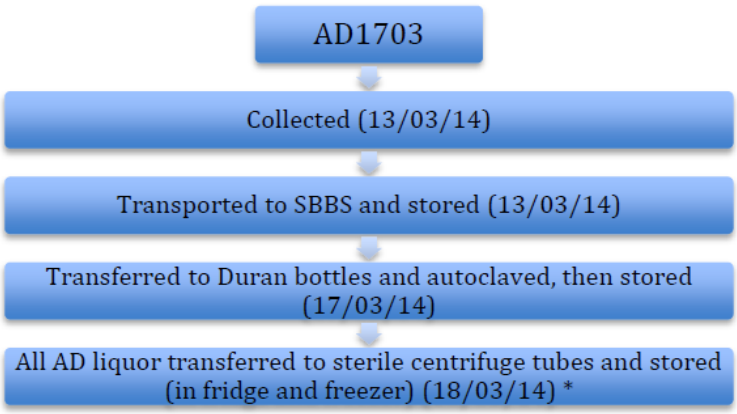

Figure 5: AD1703 collection, treatment and usage summary.

* = Process carried out in sterile conditions.

Four growth experiments used refrigerated AD1703 and one used frozen AD1703 (see Section 2.14).

2.2 Bran Sands sewage treatment works measurements

2.2.1 Ammonium, controlled oxygen demand, nitrate and phosphate

Samples were filtered through a Sartorius 90 mm diameter MGC microfibre filter paper (pore size is 1.2 µm). The concentrations of ammonium, nitrate, chemical oxygen demand and phosphate were measured using a Hach Lange DR3900 spectrophotometer according to the methods recommended by the manufacturer (Hach, 2014a, Hach, 2014b, Hach, 2014c, Hach, 2014d).

2.3 Ion chromatography analysis of ADL and growth media

Ion chromatography (IC) was carried out using a Dionex ICS3000 dual ion chromatography system. The samples were analysed using gradient analysis with distilled, deionised (DI) water and a KOH eluent (for anions) or a MSA 28 mM eluent (for cations). IC analysis was always carried out at 35 °C and anions were measured with a fitted IonPac AS19 (2 mm); cations with an IonPac CS16 (3 mm) column. This measured the concentrations of F^-, Cl^-, NO_3^-, Br^-, NO_2^-, SO_4^{2-}, PO_4^{3-}, Na^+, NH_4^+, K^+, Mg^{2+} and Ca^{2+}. The anion and cation IC instruments were calibrated every 20 samples with standard solutions (see Appendix B1). All calibrations were made using a straight line fit, with the $r^2 > 0.995$. Additionally, a range of control checks are performed, including a blank containing DI water, a liquid standard (QC) and a Certified Reference Material (CRM) sample (Lethbridg-03). The nitrate (NO_3^-) and nitrite (NO_2^-) concentrations are also measured using UV spectrophotometry (integrated with the Dionex machine) by measuring the absorbance of the solution at 210 nm and 220 nm. Appendix A2 describes the validation methods used to ensure data collected using IC was comparable to data collected at Bran Sands STW.

2.4 Selection of microalgae species

The microalgae used in this study was *Scenedesmus obliquus* (*S. obliquus*), strain SAG 276-7 from the Sammlung von Algenkulturen (SAG) (translated to culture collection of algae) of the University of Goettingen, Germany (SAG, 2013). The strain used in this study was isolated from Cambridge so is native to the UK and not genetically modified, as well as being cheap and easy to obtain (SAG, 2013). *Scenedesmus obliquus* is a common contaminant of sewage in the UK (Scott et al., 2013) and it has previously been found to work effectively at stripping nutrients from ADL over a six month period in UK climatic conditions (Scott et al., 2013; Phillippe Mozzanega, University of Bath, personal communication, 2013). The species presents a promising fatty acid profile to allow the potential production of biodiesel or as a substrate for AD (Gouveia and Oliveira, 2009). The species is quite resistant to changing conditions (Lürling, 2003), which is important if it were to be grown on unsterile ADL with changing nutrient compositions, in a changing light and temperature regime. *S. obliquus* is also able to grow at a wide range of temperatures (see Section 2.8.3). Arbib et al. (2013a) found that optimum biomass productivity is obtained at N:P ratios of 9 – 13, but the species can grow on ratios between 1:1 and 35:1 (though Flynn (2010) argued that internal C:N:P ratios and external total nutrient concentrations are the factors that affect microalgae growth, not external N:P ratios). *S. obliquus* also tolerates a wide range of pH; Goldman et al. (1982) describe *S. obliquus's* ability to grow well between pH 7.6 and pH 10.6. Guedes et al. (2011) document the species growing best at approximately pH 6 and Thielmann et al. (1990) record growth between pH 5 and pH 11. *S. obliquus* has a fast growth rate; Ho et al. (2010) recorded a growth rate of 1.19 d^{-1},Toyub et al. (2008) measured a growth rate of 0.32-0.42 μg d^{-1}; and Hodaifa et al. (2010) recorded a growth rate of 0.024 h^{-1} (or 0.576 d^{-1}). The fast growth rate and the fact it is easy to culture ((Trainor, 1998) cited by (Çelekli et al., 2008, Lürling, 2006)) means that there is a large body of literature available on this species.

2.5 Growth medium

Bold's basal medium (BBM) is a growth medium commonly used to culture *S. obliquus* (Toyub et al., 2008, Scott et al., 2013). The Culture Collection of Algae and Protozoa (CCAP) recommend the use of BBM with 3 – fold nitrogen and vitamins. For the majority of experiments the growth medium used was Bold Modified Basal Freshwater Nutrient Solution (purchased in a concentrated form from Sigma Aldrich), diluted with DI water. In Experiment 8 (see Section 2.14 for explanation of experiments performed during this study), BBM was made up according to the guidelines outlined by CCAP (no date), with added HEPES buffer to a final concentration of 20 mM. "BBM – N and P" was made up in the same manner as BBM, however the $NaNO_3$, KH_2PO_4 and $K_2HPO_4.3H_2O$ were replaced by KCl and elevated levels of NaCl. The chemical components of the three growth mediums are shown below (Table 1). The growth mediums were maintained at pH 7 using HCl. DIC availability is explained in Section 2.8.4.

Table 1: Chemical makeup of Bold Modified Basal Freshwater Nutrient Solution, Bold's Basal Medium and Bold's Basal Medium – N and P. Concentrations of Bold Modified Basal Freshwater Nutrient Solution sourced from Sigma-Aldrich (no date). Nutrient concentrations of Bold's Basal Medium and Bold's Basal Medium – N and P calculated from masses of components used when making media (see Appendix B2).

Component	Chemical Formula	Concentration (mg/l)		
		Bold Modified Basal Freshwater Nutrient Solution	Bold's Basal Medium	Bold's Basal Medium - N and P
Boric acid	H_3BO_3	11.42	11.4	11.4
Calcium chloride dihydrate	$CaCl_2.2H_2O$	25	25	25
Cobalt nitrate • $6H_2O$	$Co(NO_3)_2.6H_2O$	0.49	0.49	0.49
Cupric sulfate • $5H_2O$	$CuSO_4.5H_2O$	1.57	1.57	1.57
EDTA (free acid)	$EDTA\,Na_2$	50	50	50
Ferrous sulfate • $7H_2O$	$FeSO_4.7H_2O$	4.98	4.98	4.98
Magnesium sulfate • $7H_2O$	$MgSO_4.7H_2O$	75	75	75
Manganese chloride • $4H_2O$	$MnCl_2.4H_2O$	1.44	1.44	1.44
Molybdenum trioxide	MoO_3	0.71	0.71	0.71
Nickel chloride • $6H_2O$	$NiCl_2.6H_2O$	0.003	0	0
Potassium chloride	KCl	0	0	160
Potassium hydroxide	KOH	31	31	31
Potassium iodide	KI	0.003	0	0
Potassium phosphate monobasic	KH_2PO_4	175	175	0
Potassium phosphate dibasic	$K_2HPO_4.3H_2O$	75	75	0
Sodium chloride	NaCl	25	25	172
Sodium nitrate	$NaNO_3$	250	250	0
Sodium selenite	$Na_2SeO_3(H_2O)_5$	0.002	0	0
Stannic chloride	$SnCl_4$	0.001	0	0
Vanadium sulfate • $3H_2O$	$VOSO_4.3H_2O$	0.0022	0	0
Zinc sulfate • $7H_2O$	$ZnSO_4.7H_2O$	8.82	8.82	8.82
Sulphuric acid	H_2SO_4	0	1.77	1.77
HEPES	$C_8H_{18}N_2O_4S$	0	4766	4766

2.6 Sterile culturing of microalgae

To ensure cultures and equipment were not contaminated; aseptic work was carried out within a laminar flow hood according to SBBS standard practices.

Once the laminar flood hood had been turned on, the entire surface was sprayed with 70 % ethanol and wiped down to maximize ethanol surface contact and remove excess liquid. After excess ethanol fumes had evaporated, the Bunsen burner was lit and gloves were put on and sprayed with 70 % ethanol (outside of the laminar flow hood). Everything put into the laminar flow hood was sprayed with 70 % ethanol and every time hands were removed from the flow hood, they were re-sprayed

with 70 % ethanol. The laminar flow hood was large enough for objects that had been recently sprayed with 70 % ethanol to be separated from the Bunsen burner by 70 cm until the 70 % ethanol had evaporated.

The three main tasks carried out in the laminar flood hood were pouring and inoculating agar plates (Section 2.9), transferring autoclaved liquid to sterile centrifuge tubes/flasks and inoculating and sampling liquid cultures. In all three cases it was important that hands and arms did not pass over open cultures/plates/tubes. Additionally, only autoclaved or sterile equipment came into contact with sterile cultures/solutions. Pipette tips were autoclaved and only opened within the laminar flow hood. Sterile centrifuge tubes and serological pipettes were only opened within the laminar flow hood. Beakers and glassware were autoclaved with a lid (loose plastic lid, bung or a foil cap) and only opened within the laminar flow hood. Spreaders and inoculation loops were dipped in ethanol and flamed to remove ethanol residue.

Larger volumes of sterile liquid were transferred into sterile flasks or centrifuge tubes with an Eppendorf Easypet Electronic Pipet Aid and sterile serological pipettes. Flasks/centrifuge tubes were closed in the laminar flow hood. Smaller volumes of sterile liquid were transferred from flasks with a micropipette. First, the foil cap was removed from the flask (containing microalgae), placed in the corner of the laminar flow hood (facing downwards in a location where no other equipment had touched the surface) and the neck of the flask was flamed until the condensation was removed. The sample was then removed with the pipette (and a new pipette tip) and care was taken to ensure that the pipette tip did not touch anything except the contents of flask. The neck of the flask was flamed again before either taking a new sample or replacing the foil cap.

When work was finished in the laminar flow hood, gloves were kept on while equipment was removed. The gas tap was switched off, the work surface was sprayed with 70 % ethanol and wiped down and finally the light and airflow were switched off.

2.7 Wavelength scans

Wavelength scans were carried out using the “Scan” function on a Thermo-Scientific Genesys 10S UV-Vis Spectrometer. Disposable polystyrene cuvettes were used, with a wavelength range of 340 nm to 750 nm. Blanks were carried out with DI water.

2.8 Growth room conditions

2.8.1 Flask shaker

Figure 6: Arrangement of flasks containing *Scenedesmus obliquus* on KS501 Digital IKA Labortechnik flask shaker in growth room used in this study.

Flasks were placed on a KS501 Digital IKA Labortechnik flask shaker and set to rotate at 130 ± 2 rpm.

2.8.2 Light intensity

The lighting conditions were set to constant illumination (as recommended by members of staff at the University culturing microalgae) in the culture room. The mean light intensity was measured at the level of the surface of the flasks with a Biospherical Instruments' Quantum Scalar Laboratory (QSL-2100) radiometer and was found to be 37.7 µmol m^{-2} s^{-1}, with a standard deviation of 1.12 µmol m^{-2} s^{-1} (a relative standard deviation of 2.99 %, see Appendix B3).

2.8.3 Temperature

The temperature of the growth room was set to 25 °C. Most studies on *S. obliquus* cultivated the organism between 20 °C and 30 °C. Xu et al. (2012) found little change in growth rate between 14 °C and 30 °C (0.226±0.001 d^{-1} at 14 °C; 0.218±0.019 d^{-1} at 20 °C; and 0.243±0.010 d^{-1} at 30 °C), however the cultures in these experiments were photoinhibited so do not represent normal growth. Hodaifa et al. (2010) found that the highest specific growth rate of *S. obliquus* 276-3a (obtained from CCAP) was at 29.5 °C (0.024 hr^{-1} or 0.576 d^{-1}). Martínez et al. (1999) found that the highest specific growth rate of *S. obliquus* 276-3a (obtained from SAG) was achieved at 30 °C (0.047 h^{-1} or 1.128 d^{-1}), but the highest yield occurred at 20 °C, and Lucas-Salas et al. (2013) chose to culture this strain (CCAP 276-3a) at 22 ± 2 °C (achieving growth rates between 0.110-0.160 d^{-1}). Ho et al. (2010) and Abeliovich and Azov (1976) cultured *S. obliquus* at the relatively high temperatures of 28 °C and 30 °C respectively (Ho et al. (2010) achieved growth rates of 1.19 d^{-1} and Abeliovich and Azov (1976) did not calculate growth rates). However their strains were isolated from much warmer countries (Israel and Taiwan, respectively). The CCAP suggest Adesanya et al. (2012) a reference study of strain 276-7 (the strain used in this culture). In the reference paper, *S. obliquus* is cultivated at 25 ± 1 °C. As this temperature falls in the 20 – 30 °C window of common cultivation temperatures and 276-7 is an English strain (so could potentially have a preference for lower temperatures than subtropical strains), it seemed acceptable to culture it at 25 °C. When cultivating microalgae outside the temperature may be more variable (Craggs et al., 2012, Hulatt and Thomas, 2011, Boelee et al., 2013). However as Bran Sands has access to waste heat from the AD unit, it should be possible to control

the cultivation conditions and maintain cultures at this temperature, or any temperature below 40 °C (Andrew Moore, NWL, personal communication, 2013).

2.8.4 Culture flasks

100 ml and 250 ml Erlenmeyer flasks were used to culture the microalgae. Before cultures were inoculated, flasks were cleaned, air dried, covered with a foil cap, autoclaved and allowed to cool. After that point flasks were only opened in a laminar flow hood to ensure that the inside remained sterile. 100 ml flasks contained 30 ml of culture and 250 ml flasks contained 50 ml of culture. The flasks were not aerated, however they were only partially sealed by a foil cap and were constantly agitated by the flask shakers (according to standard practice in SBBS). This combined with the fact that the proportion of liquid in the flask was low compared to the flask volume, meant the surface area of the culture was sufficiently large enough to allow CO_2 to dissolve into the solutions, yet not so large that evaporation became an issue.

2.9 Agar plates

2.9.1 Preparing plates

Two 500 ml solutions were prepared: 500 ml of liquid media at 2× normal concentration and 15 g of agar mixed with 500 ml of DI water. Both bottles were autoclaved at 121 °C for 20 min. The agar and media were quickly mixed in a laminar flow hood, poured into plates and left to dry for 30 – 40 min.

2.9.2 Media make up

Two types of agar plates were prepared; BBM plates and lysogeny broth (LB) plates. BBM media was prepared using the Bold Modified Basal Freshwater Nutrient Solution. The LB Agar was made up by dissolving 8 g of Pepton/Tryptone, 4 g of Yeast Extract and 12 g of NaCl into 800 ml of DI water.

2.9.3 Streak plates and spread plates

Streak plates and spread plates were used to create back up cultures on agar made from BBM. Spread plates containing LB agar were also used to test for contamination.

2.9.3.1 Streak plates

First the laminar flow hood and equipment were sterilized, and then an agar plate containing *Scenedesmus obliquus* was opened. A sterilized wire inoculation loop was used to transfer a small amount of microalgae from the original plate to the new agar plate and gently dragged backwards and forwards to create a zigzag pattern.

2.9.3.2 Spread plates

The laminar flow hood and equipment were sterilized and then a flask containing a culture of *Scenedesmus obliquus* was opened. 1 ml of culture was transferred from the flask to the new agar plate using a sterile micropipette. A sterilized glass spreader was then placed on the surface of the agar and moved backwards and forwards to spread the inoculum over the surface of the agar. The plate was rotated and the process repeated until the surface of the agar was covered.

2.10 Microalgae rinsing

In Experiments 4, 6, 8 and 9 (see Section 2.14) microalgae were "rinsed" to reduce transferring nutrient residues between flasks. Firstly, a relatively large volume of culture (10-20 ml) was removed from a flask and placed in a centrifuge tube (all within a laminar flow hood). The culture was then centrifuged in a Heraeus Megafuge 2.0R centrifuge at 4000 g, at 25 °C for 10 min. The centrifuge tube was returned to the laminar flow hood and the supernatant was poured off. The microalgae pellet was then re-suspended in approximately 10 ml of autoclaved DI water. The centrifuge tube was then centrifuged again at 4000 g, at 25 °C for 10 min, prior to return to laminar flow hood and the supernatant was again poured off. The microalgae pellet was re-suspended with a smaller amount of autoclaved DI water (approximately 4-5 ml). Optical density (OD) measurements of the new culture were taken and it was then calculated what volume of this concentrated culture would be needed to inoculate the new cultures at a suitable OD.

2.11 pH measurements

The pH measurements were initially taken with pH strips (Fisherbrand pH-Fix 0-14 and Fisherbrand pH-Fix 7-14). Later measurements were taken with a Jenway 924 007 Combination electrode and a Jenway 3020 pH meter, calibrated with standard buffer solutions of pH 4.0, 7.0 and 10.0 (Oakton pH calibration buffers).

2.12 Optical density measurements

OD measurements were taken with a Unicam Helios Alpha UV/Vis Spectrophotometer. 1 ml of solution was placed in 1.5 ml polystyrene disposable cuvettes with a wavelength range of 340 nm – 750 nm (Fisher Scientific 1160 2609 cuvettes) and the absorbance of the culture was measured at 725 nm. The reasons for choosing this method to measure microalgae growth are discussed in Appendix A1.

2.13 Transmission electron microscopy of microalgae cells

Transmission electron microscopy (TEM) is a microscopy technique utilizing electrons passing through a thin section of a sample. TEM can create higher resolution images than visible light microscopy, due to the small de Broglie wavelength of electrons.

2.13.1 Chemical fixation

1 % low melting point agarose was made up with BBM and 10 % ADL. 1ml of the microalgae culture was spun in a microtube at 200 g for 1 min. The supernatant was then removed and the spun pellet was embedded into the relevant 1 % agarose solution. This was then fixed overnight in 2.5 % glutaraldehyde in 0.1 M cacodylate at pH 7.4. The pellet was gently released from the bottom microtube and post fixed for 2-4 hrs in 1 % osmium tetroxide in 0.1 M cacodylate (pH 7.4), dehydrated through alcohols and finally embedded in araldite resin.

2.13.2 Transmission electron microscopy of microalgae cells

50-70 nm sections were cut with a diamond knife using a Leica UC6 ultramicrotome. Araldite sections were stained with 2 % (w/v) uranyl acetate in ethanol for 5 min, followed by Reynolds lead citrate for 5 min. Lowicryl sections were stained with 2 % (w/v) aqueous uranyl acetate for 5 min, followed

by Reynolds lead citrate for 5 min. Sections were examined with a Hitachi H-7600 TEM operating at 100 kV.

2.14 Experimental Plans

2.14.1 Experiment 1 – Growth Assay

2.14.1.1 Objective

The aim of this experiment was to monitor microalgae growth in different dilutions of ADL (batch AD2901 diluted with DI water) to determine whether *Scenedesmus obliquus* was able to tolerate neat or diluted ADL.

2.14.1.2 Setup

The ADL concentrations chosen were 0 % (pure DI water), 1 %, 10 %, 40 %, 70 %, 100 %. Microalgae were also grown on BBM and a flask containing 100 % ADL was also monitored (100 % - M) (see Table 2). The neat AD2901 contained 1160.48 mg/l NH_4^+ (or 901.1 mg/l NH_4-N), 0 mg/l NO_3-N, 0.12 mg/l NO_2-N and 86.35 mg/l PO_4-P. The BBM contained 0 mg/l NH_4-N, 39.76 mg/l NO_3-N, 0 mg/l NO_2-N and 51.39 mg/l PO_4-P.

Table 2: Overview of Experiment 1. Column headings refer to percentage concentration of ADL in a 30 ml solution, diluted with deionized water. Each growth media solution was present in triplicate (x3). Green cells represent growth media solutions that have been inoculated with *Scenedesmus obliquus.* BBM = Bold's Basal Medium; 100%-M = 100% ADL solution that has not been inoculated with *Scenedesmus obliquus*.

0 %	1 %	10 %	40 %	70 %	100 %	BBM	100%-M
x3	x3	x3	x3	x3	x3	x3	x3
x3	x3	x3	x3	x3	x3	x3	x3
x3	x3	x3	x3	x3	x3	x3	x3

BBM = Bold's Basal Medium

= Inoculated with *Scenedesmus obliquus*

The 1 %, 10 %, 40 %, 70 % and 100 % concentrations were chosen to observe microalgae growth across a spectrum of ADL concentrations. The 0 %, 100%-M (without microalgae) and growth medium flasks were present as controls. The purpose of the 0 % controls were to record whether growth was possible when no nutrients were present, and if it was what growth rate and final cell density could be achieved. The growth medium controls were to document the maximum growth that can be achieved with ideal nutrient concentrations. The 100 % ADL containing no microalgae controls were present to test whether the optical properties of ADL change over time.

The nutrient concentrations of the ADL were tested at the beginning of the experiment with IC analysis. The dilutions of ADL and controls were prepared in triplicate. The relevant flasks were inoculated with *Scenedesmus obliquus* at an optical density of 0.1 (at 725nm in clear media).

It should be noted that in this experiment, to inoculate the algae at 0.1 OD, a certain amount of growth media containing suspended microalgae cells was needed. The actual concentrations of DI water, growth media and ADL in each flask are documented in Table 3.

Table 3: Volumetric composition of solutions in flasks used in Experiment 1. Column 1 shows the names of the flask cultures used in the experiment. The "Microalgae" in column 2 refers to a BBM solution containing a high concentration of *S. obliquus*. The remaining columns show the volume of different liquids added to different flasks (ADL = anaerobic digestate liquor; water = deionised water; BBM = Bold's Basal Medium). All liquids and flasks were autoclaved and work was carried out under aseptic conditions in a laminar flow hood.

Name	Microalgae (ml)	ADL (ml)	Water (ml)	BBM (ml)	Total Volume (ml)
BBM	3.3	0	0	26.7	30
0%	3.3	0	26.7	0	30
1%	3.3	0.3	26.4	0	30
10%	3.3	3	23.7	0	30
40%	3.3	12	14.7	0	30
70%	3.3	21	5.7	0	30
100%	3.3	26.7	0	0	30
100%-M	0	26.7	0	3.3	30

The experiment was carried out over 16 days between the 05/03/2014 and the 21/03/2013 and over this period OD measurements were taken at 725 nm every day.

2.14.2 Experiment 2 – Preliminary Adaption Test

2.14.2.1 Objective

The aim of this experiment was to check whether *Scenedesmus obliquus* had adapted to conditions in a flask containing 10 % ADL.

2.14.2.2 Setup

On the 21/03/2014, 2 flasks were inoculated with microalgae from a 10 % culture grown in Experiment 1. Both 100 ml flask contained 1 ml of microalgae culture, 3 ml of ADL (AD2901) and 26 ml of autoclaved DI water. Only two flasks had been autoclaved which is why two were used instead of three. As this was a preliminary trial, only three OD measurements were taken (at 725 nm) and the experiment was finished on the 26/03/2014.

2.14.3 Experiment 3 - Acclimation Test A

2.14.3.1 Objective

The aim of this experiment was to determine whether microalgae previously grown on 10 % ADL would have a reduced lag time when sub-cultured into new 10 % and 20 % ADL flasks. It was

theorized that if the lag time was shorter, it may indicate that the microalgae had adapted to the conditions in 10 % ADL.

2.14.3.2 Setup

Microalgae from a 10 % culture (10% B) from Experiment 2 were sub-cultured into new 10 % and 20 % ADL dilutions. Each 100 ml flask contained 30 ml of culture. 10 % flasks contained 3 ml of ADL, 2 ml of microalgae culture and 25 ml of DI water. 20 % flasks contained 6 ml of ADL, 2 ml of microalgae culture and 22 ml of DI water. Each concentration was prepared in triplicate (3 x 10 %, 3 x 20 %). 2 ml of microalgae culture was needed to ensure that the OD of the microalgae culture was 0.1 .The ADL absorbed light as well so the starting OD's were greater than 0.1. There was an issue with the fact that the microalgae were being sub-cultured from a previous 10 % culture, so a proportion of the OD signal was already due to ADL; however as all of the flasks were sub-cultured with the same amount they would be comparable with each other. AD1703 was used as it was suspected that AD2901 was contaminated. Based on visual inspection it could be noted that AD1703 was very dark brown; it was not possible to see through a 50 ml centrifuge tube containing AD1703 – while it was possible to see through a 50 ml centrifuge tube containing AD2901. The experiment was carried out between the 07/04/2014 and the 22/04/2014 and OD measurements were taken at regular intervals over this period. The pH was also measured using pH strips (the ADL had the consistency of water, so the pH strips were able to accurately measure its pH).

2.14.4 Experiment 4 - Acclimation Test B

2.14.4.1 Objective

The aim of the experiment was to test whether there was a difference in the growth of microalgae previously grown on 10 % ADL vs microalgae previously grown on growth medium.

2.14.4.1 Setup

24 flasks were prepared for the experiment. This experiment was the first to scale up by using 250 ml Erlenmeyer flasks. Six flasks contained growth medium and the other 18 contained dilutions of ADL (see Table 4). The ADL (AD1703) was centrifuged to prevent the aggregation issues that affected the OD measurements in the previous experiment. Microalgae from two different pre-incubation environments were used in this experiment. Half of the flasks were inoculated with microalgae which had previously been growing in growth medium (GrM-ad) and the other half were inoculated with microalgae previously grown in 10 % ADL (10%-ad).

The microalgae were rinsed before addition to reduce the effect of pre-existing trace elements. The nutrient concentrations of the neat ADL were tested at the beginning of the experiment with IC analysis and the nutrient concentrations of all flasks were measured on the 6th and 35th days of the experiment. The experiment was carried out over 35 days between the 23/04/2014 and the 28/05/2014. OD measurements were taken at regular intervals and the pH was measured at the beginning using pH strips and at the end using a pH meter.

Table 4: Experimental conditions of cultures grown in Experiment 3. *S. obliquus* inoculum was sourced from two cultures with differing pre-incubation conditions; one had previously been growing in Bold's Basal Medium (GrM-ad) and the other had been growing in a solution containing 10 % ADL diluted with deionized water (10%-ad). These two sets of *S. obliquus* were inoculated into Bold's Basal Medium growth medium, 1 % ADL solution, 5 % ADL solution and 10 % ADL solution (diluted with DI water). Each experimental condition was performed in triplicate (x3 flasks).

	Pre-incubation environment	
Media	**BBM Growth Medium (GrM-ad)**	**10% ADL (10%-ad)**
BBM	x3 flasks	x3 flasks
1% ADL	x3 flasks	x3 flasks
5% ADL	x3 flasks	x3 flasks
10% ADL	x3 flasks	x3 flasks

2.14.5 Experiment 5 - Agar Contamination Check

2.14.5.1 Objective

Test whether the samples were contaminated with bacteria.

2.14.5.2 Setup

On the 15/04/14 three cultures were set up to be tested for contamination. Created spread plates of 10%B, GrM and JM2. 10%B and GrM were used in Experiment 2, which both came from the same initial culture. JM2 was an older culture created in February that had been sampled a few times and then remained relatively untouched until it was used in the contamination check. Additionally blanks were used to check whether the agar or the spreader was contaminated; set aside 3 blank agar plates and 2 plates that did not have any media introduced, but did have a spreader moved across them. A spread plate testing just the growth medium Bold Modified Basal Freshwater Nutrient Solution was created on agar also made from Bold Modified Basal Freshwater Nutrient Solution, to test whether the growth medium was contaminated.

Single spread plates of a 10 % culture, neat ADL and microalgae from growth medium were created on agar made from BBM, to see if microalgae and bacteria would grow in the growth medium, or just the microalgae. Once the spread plates had been created, the plates were incubated in a 37 °C culture room. The cultures were checked after 24 hours (16/04/14), then checked periodically between 24-144 hours (16/04/14 – 21/04/14) and on the 22/04 /14 the experiment was finished. The plates containing microalgae were returned to the 25 °C growth room and the LB plates were autoclaved. Ideally the plates should have been incubated at 25 °C (the temperature of the culture room). However most bacteria that grow at 25 °C can survive at 37 °C, in fact most bacteria grow faster at this temperature.

The streak and spread plates (made with BBM agar) on which *Scenedesmus obliquus* was cultured were kept in the growth room at 25 °C and were also checked periodically for bacterial colonies.

2.14.6 Experiment 6 – 10 % Monitoring

2.14.6.1 Objective

1) Repeat a scaled down version of Experiment 4 (just 10 % ADL dilutions) to test difference in growth rate and lag time between GrM-ad and 10%-ad cultures.
2) Accurately measure pH change over time in 10 % cultures

2.14.6.2 Setup

Six 250 ml Erlenmeyer flasks were prepared, each containing 5 ml of ADL (AD1703) and 45 ml of autoclaved DI water. Microalgae from two different pre-incubation environments were used in this experiment. One half of the flasks were inoculated with microalgae which had previously been growing on growth medium (GrM-ad), the other half with microalgae previously grown on 10 % ADL (10%-ad). The culture used to inoculate the GrM-ad microalgae was "GrM-ad GrMD" from Experiment 4. The "10%-ad" flasks were inoculated one of the 10 % cultures from Experiment 4. The microalgae were rinsed before inoculation. The flasks were inoculated on the 28/05/2014 and OD and pH measurements were taken periodically until the 23/06/2014.

2.14.7 Experiment 7 - Microscopy

2.14.7.1 Objective

Observe whether the cultures were contaminated and to check whether there were morphological differences between microalgae growing in growth medium and 10 % ADL.

2.14.7.2 Setup

On the 22/07/14, 3 x 1 ml samples were taken from "GrM-ad GrM A" and "GrM-ad 10% B" (from Experiment 6) in Eppendorf tubes and centrifuged the samples in 1KA mini G for 3 minutes to concentrate the microalgae cells. The supernatant was poured off and small samples were placed on microscope slides and covered with a cover slip. A Leica DM-300 microscope was used and the microalgae were observed at x10, x100 and x400 magnification. Finally the microalgae were observed at x1000 magnification using oil immersion.

2.14.8 Experiment 8 - Buffer and Trace Elements Test

2.14.8.1 Objective

Test whether controlling the pH and the addition of trace elements would improve growth.

2.14.8.2 Setup

24 x 250 ml Erlenmeyer flasks were prepared for the experiment. Six flasks contained ADL and pure water, 6 contained ADL and growth medium, six contained ADL and modified growth medium and 6 contained growth medium and water (see Table 5 The ADL (AD1703) was centrifuged being added to the flasks.

The nutrient composition of the growth medium (BBM) and modified growth medium (BBM –NP) used in this experiment can be seen in Section 2.5. Additionally HEPES buffer was added to each solution to ensure that the pH was buffered and stayed below 8. Microalgae from two different pre-

incubation environments were used in this experiment. Half of the flasks were inoculated with microalgae, which had previously been growing in growth medium (GrM-ad) and the other half were inoculated with microalgae that had previously been growing in 10 % ADL (10%-ad). Both cultures came from Experiment 6. The microalgae were rinsed before inoculation.

Table 5: Volumetric composition of growth media solutions used in Experiment 8. Each flask contained 60 ml of liquid growth media, made up from a combination of deionized water (DI H_2O), anaerobic digestate liquor (ADL), Bold's Basal Medium (BBM) or modified Bold's Basal Medium with no added nitrate or phosphate (BBM-NP). All flasks and liquids were sterilized before use and work was carried out under aseptic conditions in a laminar flow hood.

	Volume of Liquid (ml)			
	DI H_2O	**ADL**	**BBM**	**BBM-NP**
H_2O-AD	54	6	0	0
BBM-AD	0	6	54	0
BBM(-NP)-AD	0	6	0	54
BBM-H_2O	6	0	54	0

The flasks were inoculated on the 31/07/2014 and OD and pH measurements were taken at regular intervals (either 24 hr or 48 hr) until the 13/08/2014. On the 31/07/2014 and the 13/08/2014, 10 ml samples were taken each of the 24 flasks, filtered to 0.2 µm and tested with IC analysis.
Additionally TEM analysis was carried out on 5 samples. The initial two starting cultures were sent to be tested on 31/07/2014. On the 13/08/2014 "GrM-ad H_2O-AD B", "GrM-ad BBM (-NP)-AD B" and "GrM-ad BBM-H_2O B" were sent for analysis.

Photos of the flasks containing microalgae were taken on the 13/08/2014 on a Samsung Galaxy Y GT-S5360 smartphone (2 MP camera). A photo of microalgae samples left to settle in cuvettes was taken on the 15/08/2014 and the exposure of the photo was increased to better show the colour changes. The original photo is shown in Appendix B9.

2.14.9 Experiment 9 – Unsterile Culturing Experiment

2.14.9.1 Objective

1) Test whether the pH could be brought down using frozen CO_2 and remain low due to microalgae buffering/natural buffering of ADL
2) Test whether microalgae could grow successfully in unsterile conditions
3) Test whether use of tap water instead of DI water improved growth through addition of trace elements
4) Test whether microalgae could grow in concentrations of ADL > 10 % if the pH was below 8.

2.14.9.2 Setup

9 non-sterile 250 ml Erlenmeyer flasks were used in this experiment. Each flask contained 50 ml of liquid. 3 dilutions of ADL were prepared in 500 ml Duran bottles; 20 %, 60 % and 100 % ADL. The ADL was diluted with unsterile tap water instead of sterile DI water. The ADL used (AD1703) had

been frozen and then defrosted. On the 21/08/2014, pieces of frozen carbon dioxide (each weighing 2.52±0.30 g, n= 4) were added to the 20 %, 60 % and 100 % solutions of ADL to lower the pH (see Table 6).

Table 6: pH adjustment of ADL dilutions. Solutions contained differing concentrations of ADL (20, 60 and 100%) diluted with unsterilized tap water. Frozen CO_2 was added to these solutions, with the pH being measured before and after the CO_2 addition.

ADL concentration	Initial pH	No. of CO_2 blocks	pH after CO_2 addition
20%	9.21	2	6.91
60%	9.25	8	6.91
100%	9.26	11	6.96

On the 22/08/2014 the solutions were added to the flasks; 3 flasks contained 20 % ADL, 3 contained 60 % ADL and 3 contained 100 % ADL. Microalgae previously grown in growth medium (did not record origin flask of the microalgae) were inoculated into the new flasks. The microalgae were rinsed before inoculation. Inoculation was carried out on the bench (not in a laminar flow hood), partially due to lack of availability of laminar flow hoods and partially because it was an unsterile experiment. OD and pH measurements were taken at regular intervals until the particles in the solutions made it impractical to measure OD, after which only pH measurements were taken. Samples were taken on the bench, not in a laminar flow hood, with unsterile pipette tips. However the micropipette was still sterilised with 70 % ethanol.

Once it became apparent that the pH increased above 8, the pH was lowered again by dissolving $NaHCO_3$ into the solutions. $NaHCO_3$ was used instead of frozen CO_2 as it was less harmful to microalgae in solutions and potentially had more buffering capability. The amount of $NaHCO_3$ was sufficient to reduce the pH from 9 to 8 (see Figure 41), however the exact mass was not measured. The experiment was finished after 6 days on the 28/08/2014 after the pH increased above 8 once more.

2.15 Molecular weight conversions

Molecular weight conversions (e.g. between NH_3-N and NH_3) can be found in Appendix B6.

2.16 Standard errors

Standard errors are shown in tables in Appendix D. The decision to place them in the appendix rather than on figures was due to the fact that there is a large amount of data presented on many of the figures, which became difficult to interpret once error bars had been added. E.g. Figure 10 shows only 8 growth curves; however there are 88 data points on the graph, which become indistinguishable when 88 sets of error bars are displayed on the graph as well.

2.17 Ammonia concentration calculation

Free ammonia (NH_3-N) concentrations and NH_3 : NH_4 ratios were calculated according to the guidelines in FDEP (2001). In Figure 27 (showing calculated ammonia concentration plotted against pH), no NH_3-N value was calculated at a pH greater than 9.20, as the pKa (at 25 ˚C) is 9.24, so the calculations become unreliable at pH greater than this figure.

3. Results

3.1 Nutrient variation between batch samples and growth medium

The ion concentrations in Bold's Basal Medium and three batches of undiluted ADL (AD1, AD2901 and AD1703) are shown in Figure 7, Figure 8 and Figure 9. These graphs show the differences and similarities in ion concentrations between the undiluted ADL and a growth-promoting artificial medium (Bold's Basal Medium), an important exercise as some of the ions are essential nutrients and others can act as toxins. It also shows ion concentration variation between the three batches of ADL used in this study.

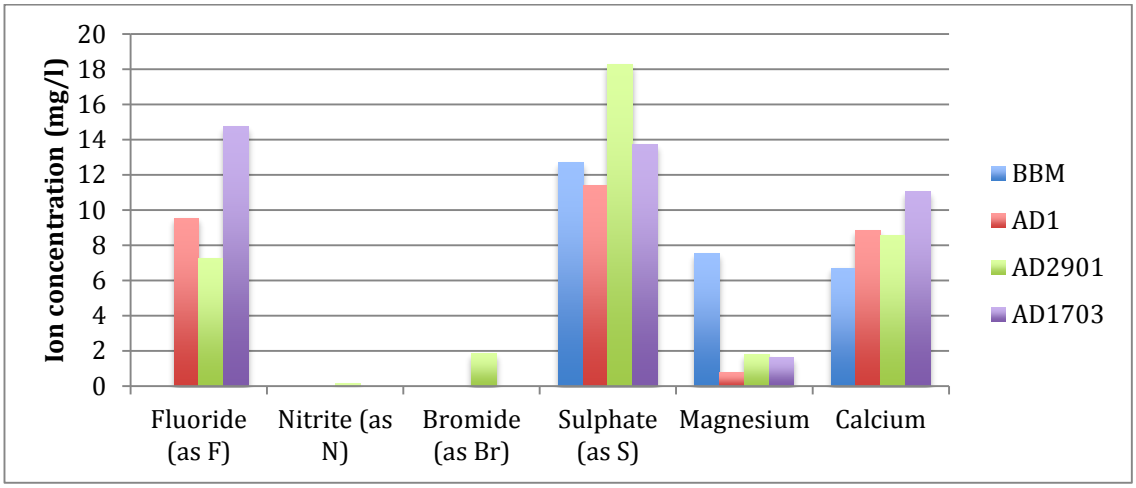

Figure 7: Fluoride (as F), nitrite (as N), bromide (as Br), sulphate (as S) magnesium and calcium ion concentrations present in Bold's Basal Medium (BBM) and three undiluted ADL samples (AD1, AD2901 and AD1703). Concentrations of BBM and AD1 represent single measurements. AD2901 and AD1703 show mean ion concentration calculated from 5 and 3 measurements respectively (standard errors shown in Appendix D1).

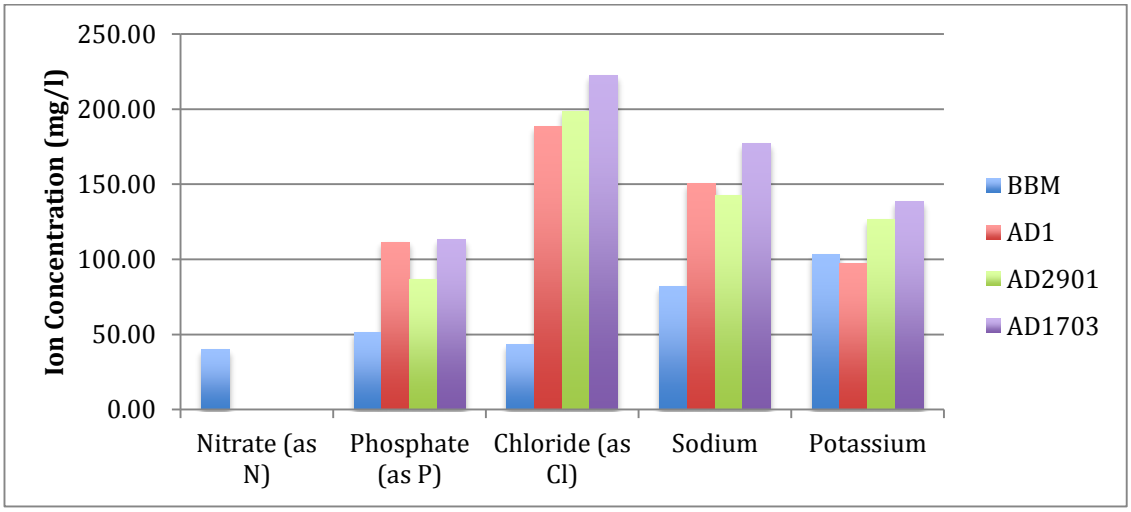

Figure 8: Nitrate (as N), Phosphate (as P), Chloride (as Cl), sodium and potassium ion concentrations present in Bold's Basal Medium (BBM) and three undiluted ADL samples (AD1, AD2901 and AD1703). BBM and AD1 data was taken from single measurements. AD2901 and AD1703 show mean ion concentration calculated from 5 and 3 measurements respectively (standard errors shown in Appendix D1).

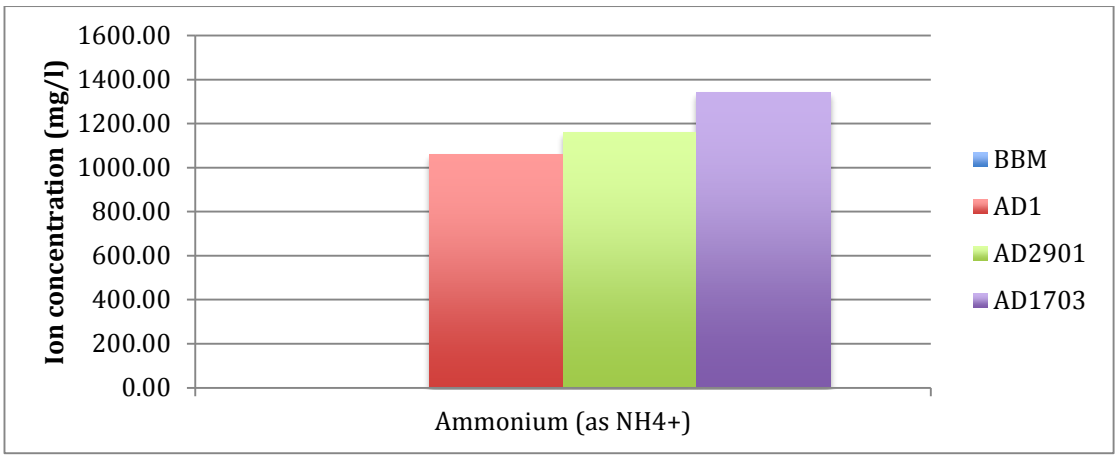

Figure 9: Ammonium (as NH_4^+) ion concentrations present in Bold's Basal Medium (BBM) and three undiluted ADL samples (AD1, AD2901 and AD1703). BBM and AD1 data was taken from single measurements. AD2901 and AD1703 show mean ion concentration calculated from 5 and 3 measurements respectively (standard errors shown in Appendix D1).

The values in Figure 7, Figure 8 and Figure 9 are means of multiple ADL samples (except in the case of BBM and AD 1, where there was only 1 sample analyzed with IC). AD1703 has the highest levels of fluoride, calcium, chloride, phosphate, sodium, potassium and ammonium. AD2901 has the highest levels of nitrite, bromide, sulphate, magnesium and potassium. There is variation between samples; the largest variation is seen in fluoride (relative standard deviation of 40 % - Appendix D1). Sulphate and magnesium also have large relative standard deviations (20 % and 33 % respectively – Appendix D1). However all other nutrients have relative standard deviations less than 14 % (Appendix D1). The potential impact of the concentrations of these ions on microalgae growth is discussed in Sections 4.2.5 and 4.2.6.

3.2 Experiment 1 – Growth Assay

3.2.1 Optical density measurements

In Experiment 1, the largest amount of biomass was generated in the growth medium. Out of the ADL dilutions, the fastest growth rate (see Figure 58 in Appendix F) and highest yield were recorded in the 1 % solution. No microalgae grew in any other dilutions of ADL, except for the 10 % solution after a 10-day lag. It should be noted that although it appears that growth is starting to happen in the 40 % flasks as well, this is actually due to one flask developing aggregates/particulate matter. These particles were not green and only occurred in one flask (possibly due to contamination).

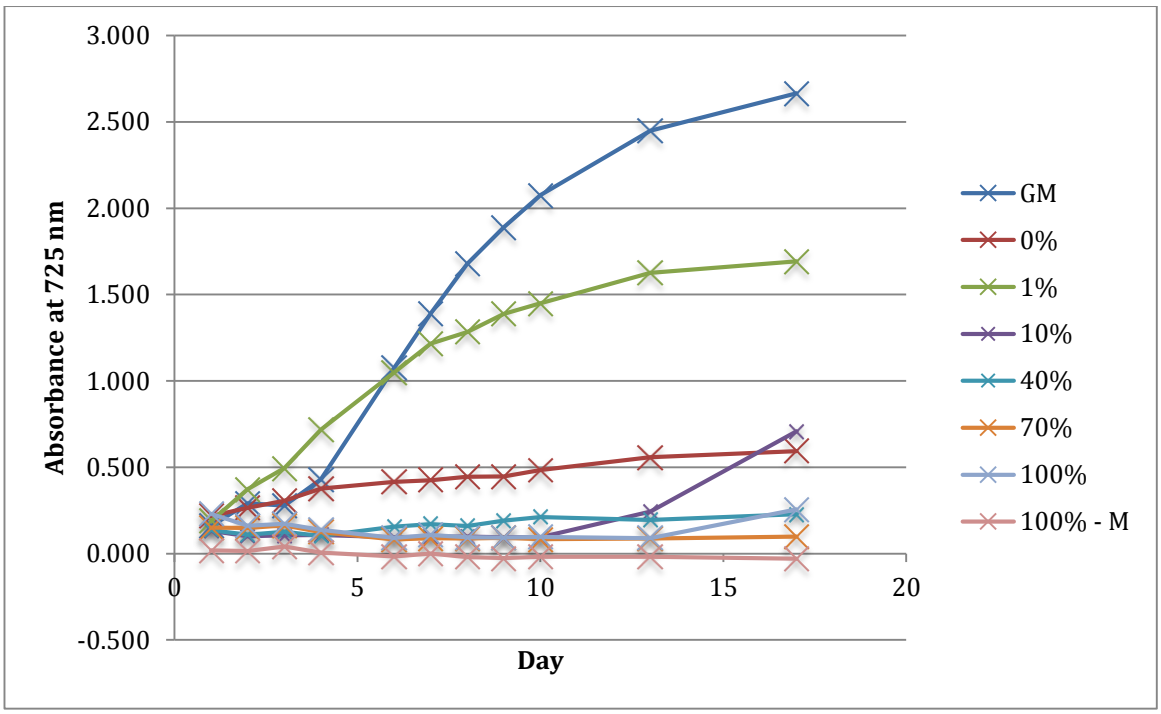

Figure 10: Mean OD measurements of neat ADL and *Scenedesmus obliquus* growing in ADL dilutions and BBM. GM = growth medium, i.e. BBM. 100%-M = 100 % ADL solution that has not been diluted with DI water or inoculated with *S. obliquus.* Each data point represents the averaged result of three replicate flasks; standard error shown in Appendix D2.

It is unclear from OD measurements alone whether microalgae have died in the flasks where no growth is observed, or simply entered a dormant state and thus haven't grown. In the flasks containing 10-100 % ADL and microalgae - none of the optical densities returned to one that you would expect to show pure ADL (0 on the normalized graph). Therefore, either the initial microalgae cells altered the optical properties of the ADL (e.g. caused it to form aggregates) then died, or a base population is present but its growth is inhibited. The observation that the microalgae in 10 % ADL suddenly entered the exponential growth phase after 11 days supports the latter explanation.

3.2.1.1 Flocculation

On the second day of the trial the microalgae in all of the flasks flocculated for an unknown reason (potentially pH changes). The flocculation had mostly disappeared in the flasks containing ADL by the third day and the cultures were no longer flocculated by the fourth day. There was a one-day lag with the microalgae in the growth medium, with the flocs remaining on the third day and starting to disaggregate on the fourth day. When observed at a × 100 magnification under a microscope on day 1 of the experiment, it could be seen that the microalgae had clustered together in flocs, not in chains. However, when viewed again on day 2, the microalgae flocculation had decreased and the culture mainly consisted of single cells, rather than circular aggregates of cells. Additionally, the ADL has shown a tendency to form aggregates. The size and colour of these aggregates typically depended on the amount of ADL present in the flask. Less ADL lead to exclusively small pale white aggregates, while higher levels of ADL (> 70 %) also contained larger brown aggregates. The flocculation event may have been caused by a change in pH when the microalgae were sub-cultured.

3.2.2 Ion chromatography analysis

The ion concentrations of the AD2901 used in this experiment were similar to those of AD2901 shown in Figure 7, Figure 8 and Figure 9 (see Appendix B8).

3.3 Experiment 2 – Preliminary Adaption Test

The OD measurements of the 10 % ADL cultures in Experiment 1 and the two 10 % ADL cultures in Experiment 2 are shown in Figure 11. The microalgae grown of the 10 % ADL in Experiment 2, were sub-cultured from one of the Experiment 1 10 % ADL cultures. Few measurements were taken for this experiment; however the data shows that the lag time in Experiment 2 (approximately 100 h) was shorter than the lag time in Experiment 1 (approximately 200 h). So while the lag was still present, it had decreased dramatically and this appeared to support the theory that the microalgae had adapted to the conditions in the ADL.

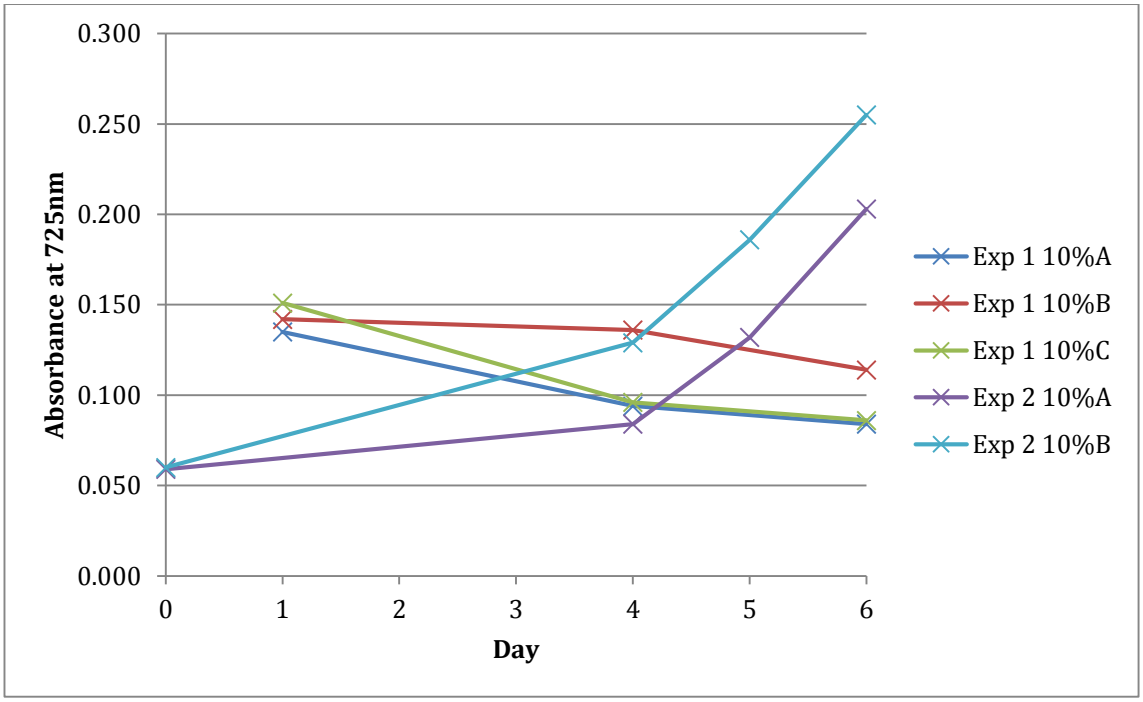

Figure 11: Comparison of OD measurements of *Scenedesmus obliquus* growing in five 10 % ADL cultures; three flasks in Experiment 1 (Exp 1 10 %) and two flasks in Experiment 2 (Exp 2 10 %), between hour 0 and 160. The *S. obliquus* used in experiment 2 were sub-cultured from one of the Experiment 1 10 % cultures.

3.4 Experiment 3 - Acclimation Test A

3.4.1 Optical Density measurements

Unfortunately, the high suspended solids content of the ADL led to the particles aggregating and affecting the OD measurements (Figure 12). This process started to happen in some flasks by day 1 (hour 48), and all flasks were eventually affected by it. By day 5 (hour 120) the aggregates were dramatically affecting the OD readings. It is unclear what caused these aggregates to appear and possibilities include pH changes or the presence of other microorganisms.

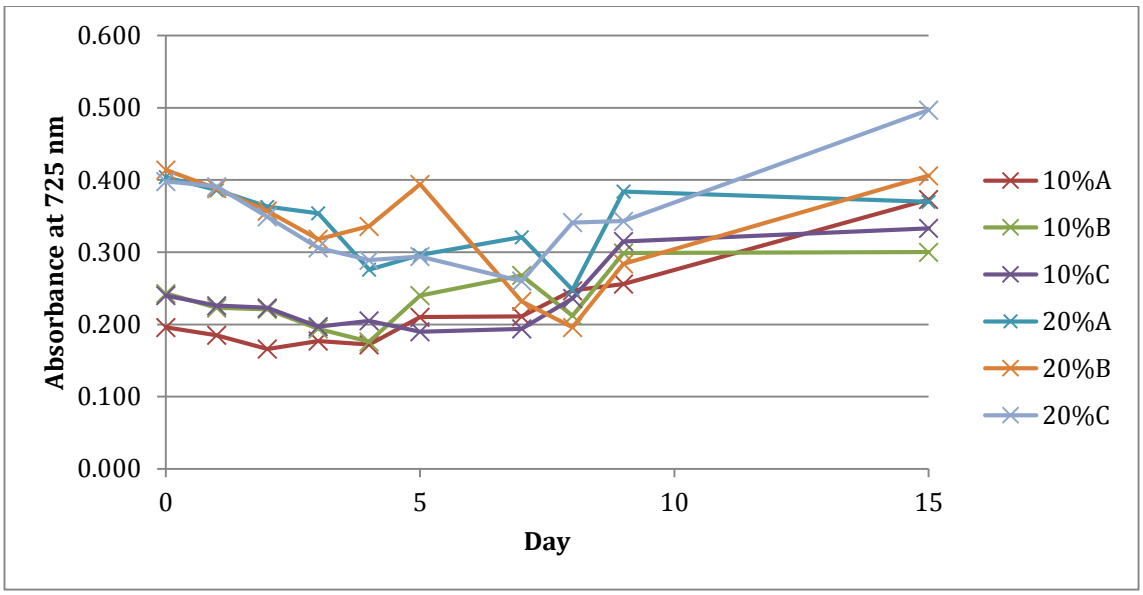

Figure 12: OD measurements of *Scenedesmus obliquus*, previously grown in a 10 % ADL solution (diluted with DI water), sub-cultured into 10 % and 20 % ADL dilutions (diluted with DI water).

The greater concentration of suspended solids in AD1703 (compared to AD2901) made the formation of aggregates more favorable, so it was not possible to take accurate OD readings to measure the growth of microalgae. Centrifuged ADL was used in later experiments to prevent this issue with aggregation from inhibiting the OD measurements.

3.4.2 pH measurements

As pH was considered a possible cause of flocculation in Experiment 2, the pH was measured in Experiment 3 (using pH strips). Figure 13 shows that the pH only started to be measured at 97 hours, by which point the pH of the 20 % flasks were 8.50-9.00 and the 10 % flasks were 8.00-8.50. The pH decreased over the course of the experiment (except in 20 % B which stays constant) and by the end of the experiment the 20 % flasks had pH values ranging from 8.0-8.5 and the 10% flasks had pH values between 7.50-8.00. It should be noted that the pH strips were less accurate than the pH meter used in later experiments.

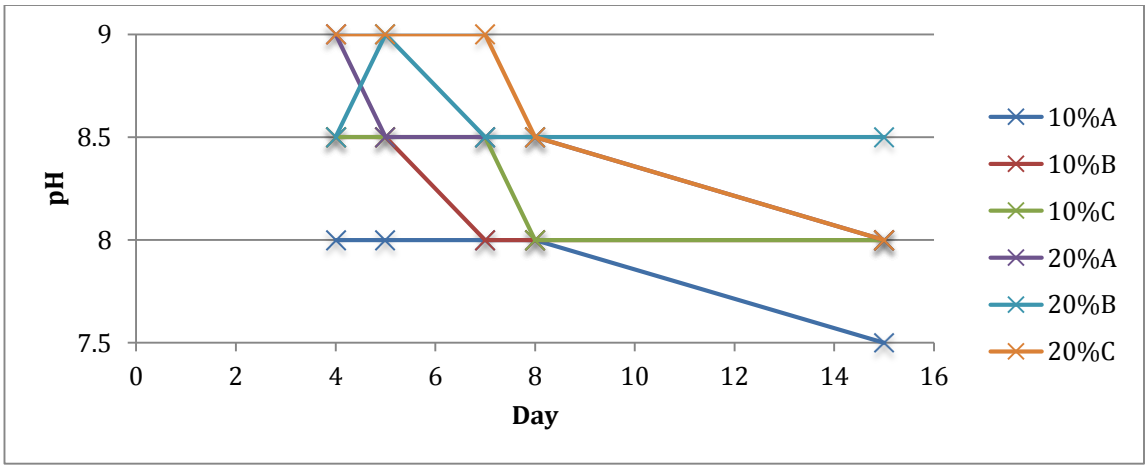

Figure 13: pH measurements of *Scenedesmus obliquus*, previously grown in a 10 % ADL solution (diluted with DI water), sub-cultured into 10 % and 20 % ADL dilutions (diluted with DI water).

3.5 Experiment 4 - Acclimation Test B

3.5.1 Optical Density measurements

In Experiment 1, most biomass accumulated in the cultures growing on growth medium and there is little difference between the OD measurements of GrM-ad GrM and 10%-ad GrM cultures (Figure 14). The highest growth rates were also recorded in BBM cultures (0.859 d^{-1} and 0.591 d^{-1} on day 2 in GrM-ad GrM and 10%-ad GrM respectively) and the growth rates remained consistently higher than the cultures containing ADL (Figure 61, Appendix F). The exception to this trend was GrM-ad 1%; on day 2 and 3 the growth rates were the same as the 10 %-ad GrM culture (0.591 d^{-1}) and GrM-ad GrM culture (0.237 d^{-1}) respectively. However after day 3 the growth rate declined in the GrM-ad 1% culture while the growth rates in BBM cultures remained higher.

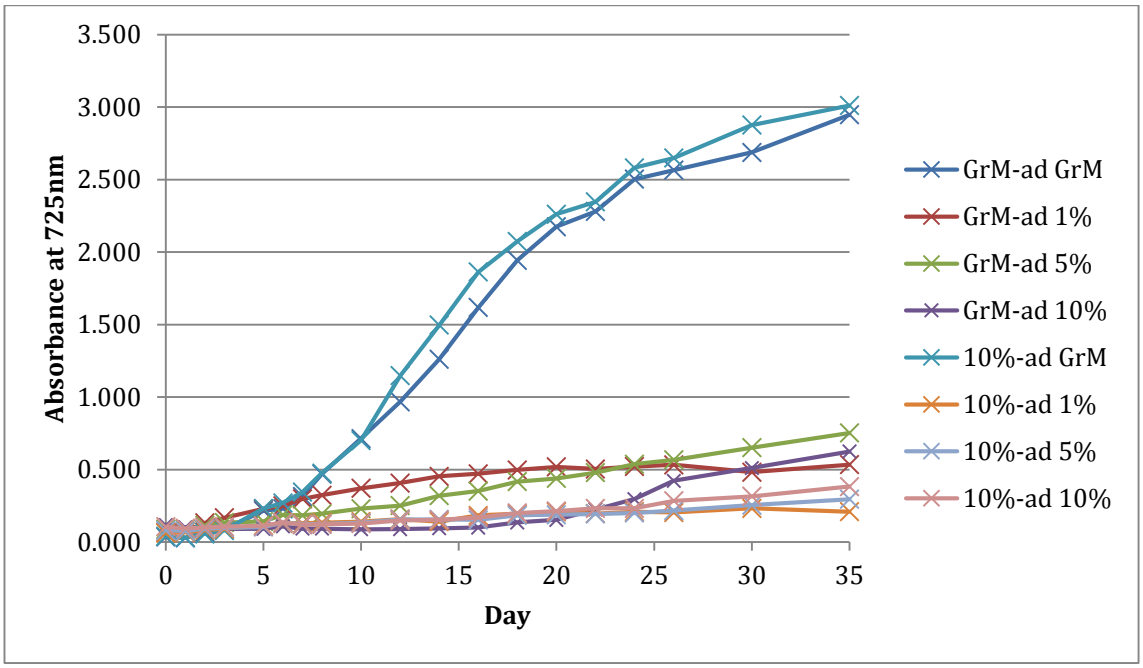

Figure 14: OD measurements of *Scenedesmus obliquus* previously grown on BBM (GrM-ad) and 10 % ADL (10%-ad) solutions, sub-cultured into BBM (GrM) and 1 %, 5 % and 10 % ADL solutions. Each data point represents the mean of triplicate measurements and the standard errors shown in Appendix D5.

GrM-ad 10% cultures are the only ones to have a lag greater than 100 hours (lasts for 400 hours), which can be observed in Figure 15. Inhibited growth occurs in all cultures grown on ADL dilutions from an early stage. Overall the GrM-ad cultures generate more biomass and have faster growth rates than the 10%-ad cultures.

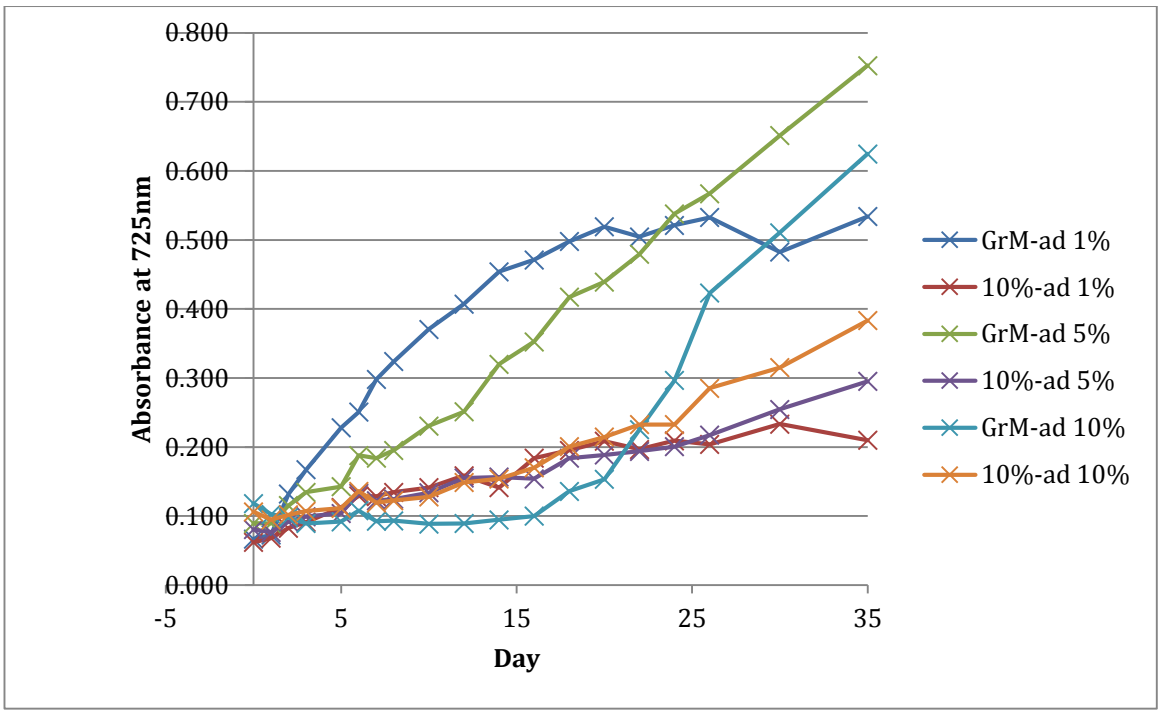

Figure 15: OD measurements of *Scenedesmus obliquus* previously grown on BBM (GrM-ad) and 10 % ADL (10%-ad) solutions, sub-cultured into 1 %, 5 % and 10 % ADL solutions (diluted with DI water). Each data point represents the mean of triplicate measurements and standard errors are shown in Appendix D5.

3.5.2 pH measurements

The mean pH measurements taken in Experiment 4 are shown in Figure 16. The first three measurements were taken with pH paper strips. However this was stopped to conserve culture volume. The final two measurements were taken with a micro-pH probe. Although accurate measurements were not taken in the early stages, and a large amount of data in the middle of the experiment is missing, it is still possible to identify broad trends. The Bold's Basal Medium flasks started with a low pH of 7.00 and increased to between pH 8.75-9.00 by the end of 800 hours. This was likely to do with the consumption of nitrate (or dissolved inorganic carbon) increasing pH and potentially the consumption of bicarbonate once the pH was greater than 8.00 – see Section 4.4.3. In contrast, the ADL dilutions started with high pH and decreased over the course of the experiment. The greater the dilution of the digestate, the lower the starting pH. The 10 % flasks had a mean pH of 8.50, the 5 % flasks had a mean pH of 8.25 and the 1 % flasks had a mean pH of 7.50 and 7.33 (in the GrM-ad and 10%-ad flasks respectively). Both GrM-ad and 10%-ad flasks have similar growth profiles with regards to OD (Figure 14) and pH change (Figure 16).

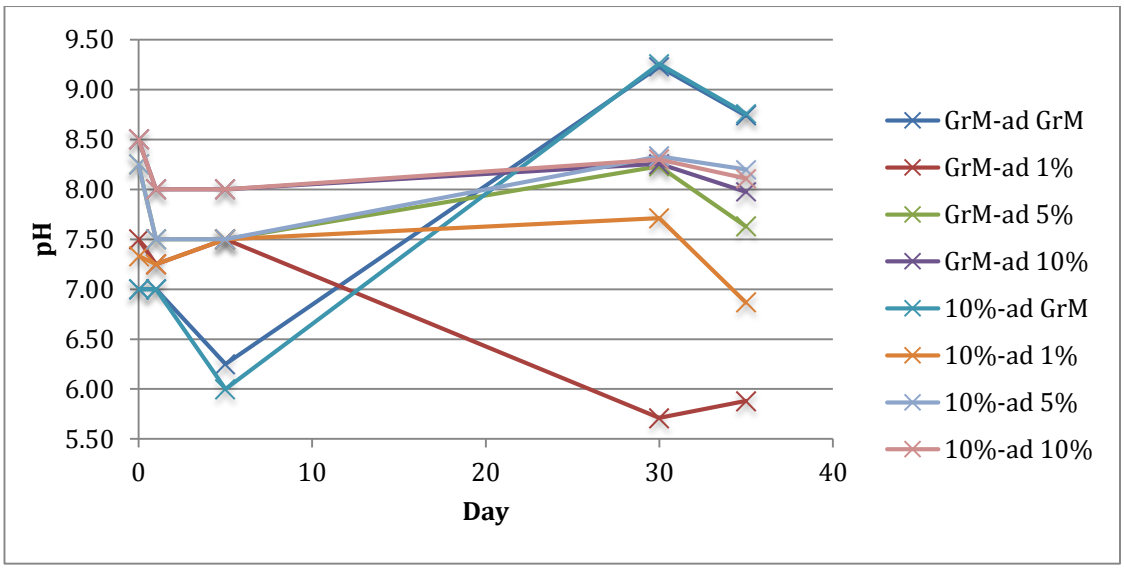

Figure 16: pH measurements of *Scenedesmus obliquus* previously grown on BBM (GrM-ad) and 10 % ADL (10%-ad) solutions, sub-cultured into BBM (GrM) and 1 %, 5 % and 10 % ADL solutions (diluted with DI water). Each data point represents the mean of triplicate measurements and standard errors are shown in Appendix D5. pH measurements between days 0-5 were taken with pH strips, after which a pH meter was used.

The GrM-ad and 10%-ad 1 % flasks show the greatest differences in both OD and pH measurements, with the GrM-ad 1 % microalgae growing more rapidly and the pH decreasing by a greater amount than the 10%-ad 1% microalgae. In the 5 % and 10 % flasks, the GrM-ad and 10%-ad microalgae have similar pH profiles, with the pH decreasing slightly more in the GrM-ad flasks by the end of the experiment. There is a broad trend that if more ammonium was consumed (Figure 19, Figure 21 and Figure 23) the pH was lower (discussed in Section 4.4.3.2), which was expected as ammonium is basic.

3.5.3 Ion chromatography analysis

An untreated sample of Bold's Basal Medium (a nitrate-based artificial growth medium) and ADL were analysed using an ion chromatographer on day 0. Concentrations in the various flasks were calculated from these values. IC analysis was carried out on every sample on day 6 and day 35, post start of experiment.

3.5.3.1 Ion chromatography results of flasks containing growth medium

In Experiment 8, 6 of the flasks (2 sets of triplicates) contained BBM as a substrate and the mean ion concentrations are shown in Figure 17 and Figure 18. It can be seen that nitrate concentrations become depleted over the 35 days. Sodium concentrations increase and the chloride and potassium concentrations fluctuate.

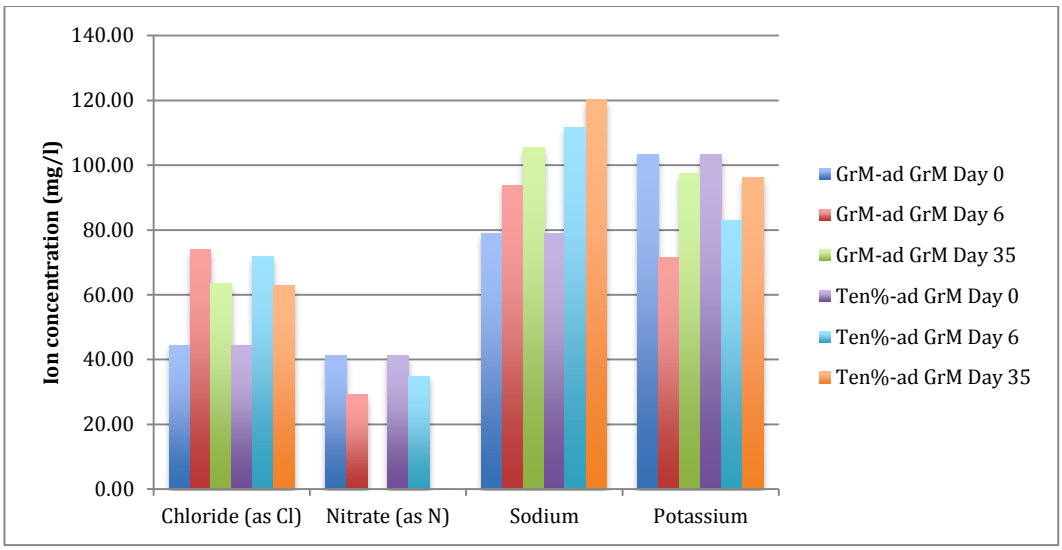

Figure 17: Mean chloride (as C), nitrate (as N), sodium and potassium ion concentrations of Bold's Basal Medium (GrM) which contained *Scenedesmus obliquus*, measured on day 0, 6 and 35 of experiment 4. The *S. obliquus* used in this experiment had previously been growing in two different growth media: GrM-ad refers to *S. obliquus* previously grown in BBM while 10%-ad refers to *S. obliquus* previously grown on a 10% ADL solution before being sub-cultured into the solutions used in experiment 4. Each data point represents the mean of triplicate measurements and the standard errors are shown in Appendix D5.

Negligible nitrite, bromide and ammonium concentrations are shown in Figure 18. Phosphate concentrations decreased but were not depleted, illustrating that these cultures were N-inhibited. Sulphate, magnesium and calcium concentrations decreased but were not depleted. Fluoride concentrations fluctuated. There was little difference in uptake patterns between GrM-ad and 10%-ad cultures.

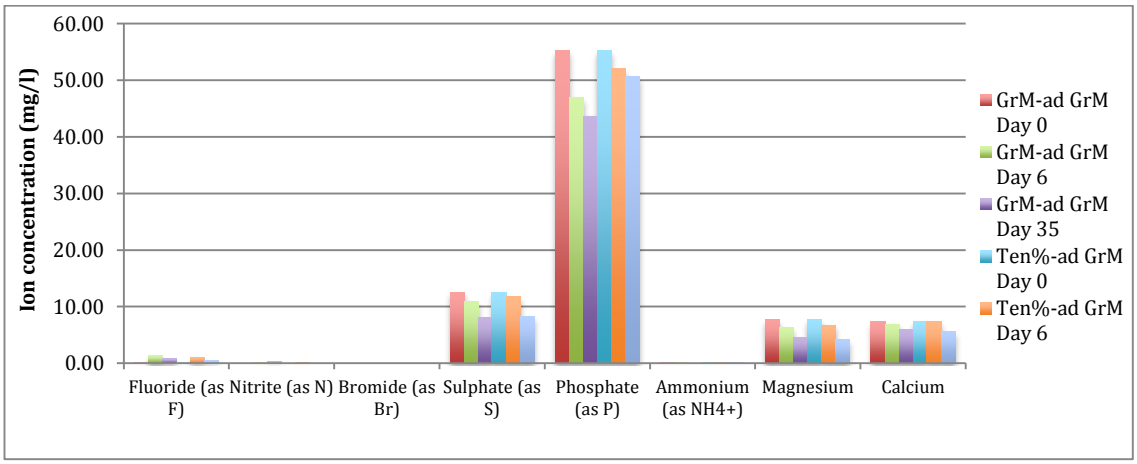

Figure 18: Mean fluoride (as F), nitrate (as N), bromide (as B), sulphate (as S), phosphate (as P), ammonium (as NH_4^+), magnesium and calcium ion concentrations of Bold's Basal Medium (GrM) which contained *Scenedesmus obliquus*, measured on day 0, 6 and 35 of experiment 4. The *S. obliquus* used in this experiment had previously been growing in two different growth media: GrM-ad refers to *S. obliquus* previously grown in BBM while 10%-ad refers to *S. obliquus* previously grown on a 10% ADL solution before being sub-cultured into the solutions used in experiment 4. Each data point represents the mean of triplicate measurements and the standard errors are shown in Appendix D5.

3.5.3.2 Ion chromatography results of flasks containing 1 % ADL

By comparing Figure 17, Figure 18 and Figure 19 to Figure 9 it can be observed that the ion concentrations shown on Day 0 in the 1 % ADL flasks were what we would expect, as they were approximately 1 % of the values measured in undiluted ADL. Figure 19 shows that ammonium concentrations decreased over the 35 days. This occurred faster in the GrM-ad cultures than in the 10%-ad cultures and the GrM-ad cultures were N-limited by day 35. Both sodium and chloride concentrations showed a tendency to increase over time, with anomalously large concentrations recorded on Day 6 in the GrM-ad cultures. The large peaks were measured in all three GrM-ad cultures, and chloride and sodium have standard errors of 0.74 and 3.19 respectively (Appendix D5). This means the peaks were not due to one anomalously large measurement. The sodium and chloride concentrations were measured on separate machine runs (cations and anions were measured separately). The sodium peak may be due to the fact that the sample was not measured on an optimal dilution; instead an estimate was made from the first neat run, which could affect the reliability of the measurements. However the chloride concentrations were measured on an optimal dilution. It is clear that the high Na and Cl concentrations are a real effect, however it was not possible to study the causes of these anomalously high ion concentrations during this project (see Appendix G).

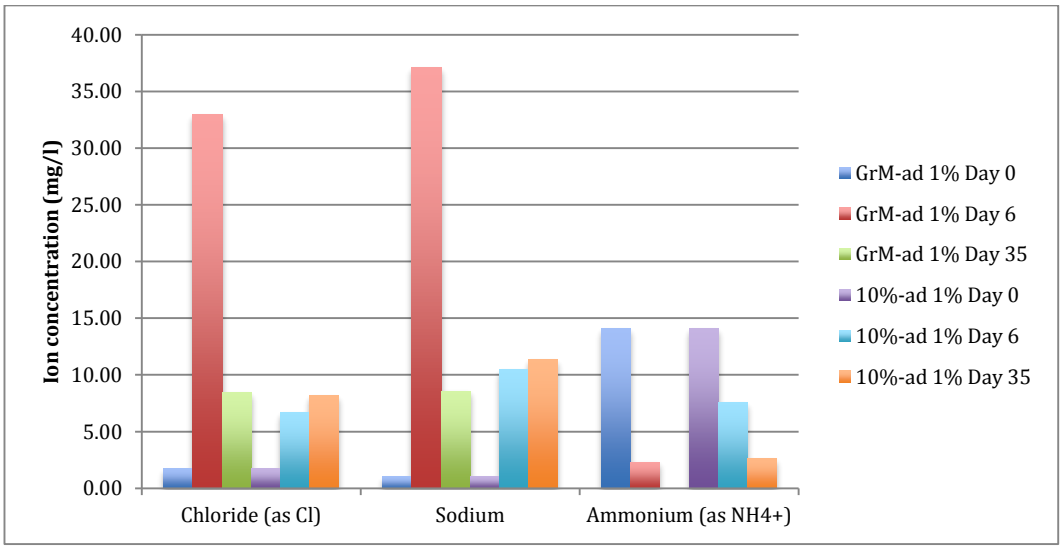

Figure 19: Mean chloride (as C), sodium and ammonium (as NH_4^+) ion concentrations of 1 % ADL solutions which contained *Scenedesmus obliquus*, measured on day 0, 6 and 35 of experiment 4. The *S. obliquus* used in this experiment had previously been growing in two different growth media: GrM-ad refers to *S. obliquus* previously grown in BBM while 10%-ad refers to *S. obliquus* previously grown on a 10% ADL solution before being sub-cultured into the solutions used in experiment 4. Each data point represents the mean of triplicate measurements and the standard errors are shown in Appendix D5.

In Figure 20, phosphate concentrations decreased in GrM-ad 1% cultures to negligible concentrations by day 35, meaning that the cultures were potentially P-limited (depending on the internal P reserves of the microalgae cells). However in the 10%-ad 1% cultures, phosphate concentrations dipped on day 6 and then dramatically increased. Nitrate, magnesium and sulphate concentrations increased. This may have been due to nutrient release from cell death, or pH change affecting the solubility of mineral phases. Bromide concentrations were negligible and nitrite

concentrations were mostly negligible except for the 10%-ad 1% cultures on Day 35. Calcium concentrations increased in GrM-ad 1% cultures and fluctuated in 10%-ad 1% cultures. Potassium concentrations fluctuated (in an inverse manner to cultures grown in growth medium). Fluoride concentrations steadily increased in 10%-ad 1% cultures, however in GrM-ad 1% cultures they increased dramatically by day 6 and then decreased by day 35.

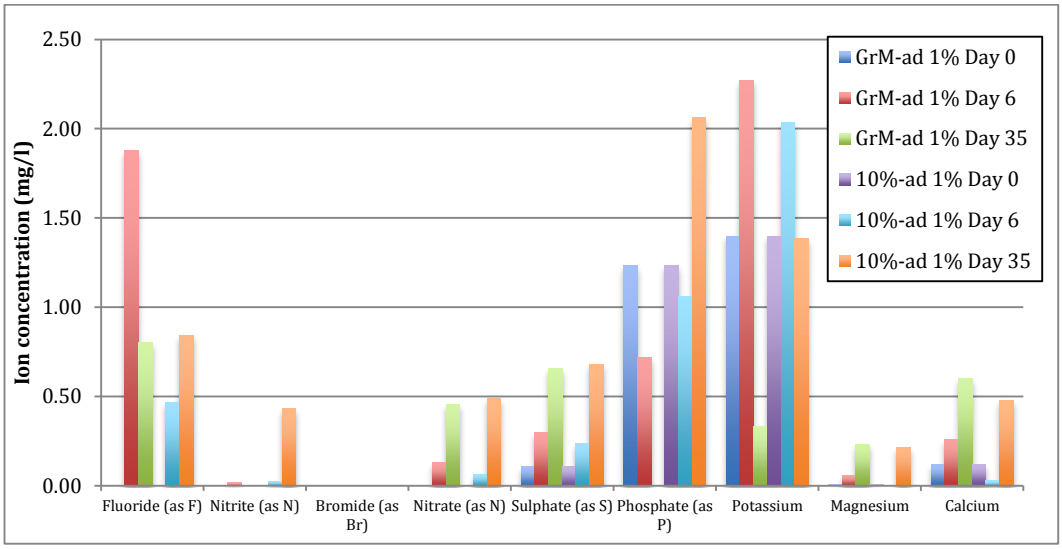

Figure 20: Mean fluoride (as F), nitrate (as N), bromide (as B), nitrate (as N), sulphate (as S), phosphate (as P), potassium, magnesium and calcium ion concentrations of 1 % ADL solutions which contained *Scenedesmus obliquus*, measured on day 0, 6 and 35 of experiment 4. The *S. obliquus* used in this experiment had previously been growing in two different growth media: GrM-ad refers to *S. obliquus* previously grown in BBM while 10%-ad refers to *S. obliquus* previously grown on a 10% ADL solution before being sub-cultured into the solutions used in experiment 4. Each data point represents the mean of triplicate measurements and the standard errors are shown in Appendix D5.

3.5.3.3 Ion chromatography results of flasks containing 5 % ADL

The chloride and sodium concentrations displayed in Figure 21 show similar patterns to those seen in Figure 19, where concentrations increased over the duration of the experiments and anomalously large concentrations were measured on Day 6 in the GrM-ad cultures. Ammonium concentrations decrease in GrM-ad 5% cultures but are not depleted as in 1 % cultures. In the 10%-ad 5% cultures the ammonium concentration increased between day 0 and 6, then decreased by day 35. There is a chance that differences in nutrient concentrations and thus growth rates, may be due to different starting conditions. All microalgae were washed twice before being added to cultures, however as digestate contains particulate matter, there is a chance that when the 10%-ad cultures were added, extra nutrients were added. However, when nutrient concentrations were taken at the beginning of Experiment 8 (Figure 30 to Figure 32) this rinsing was shown to be effective.

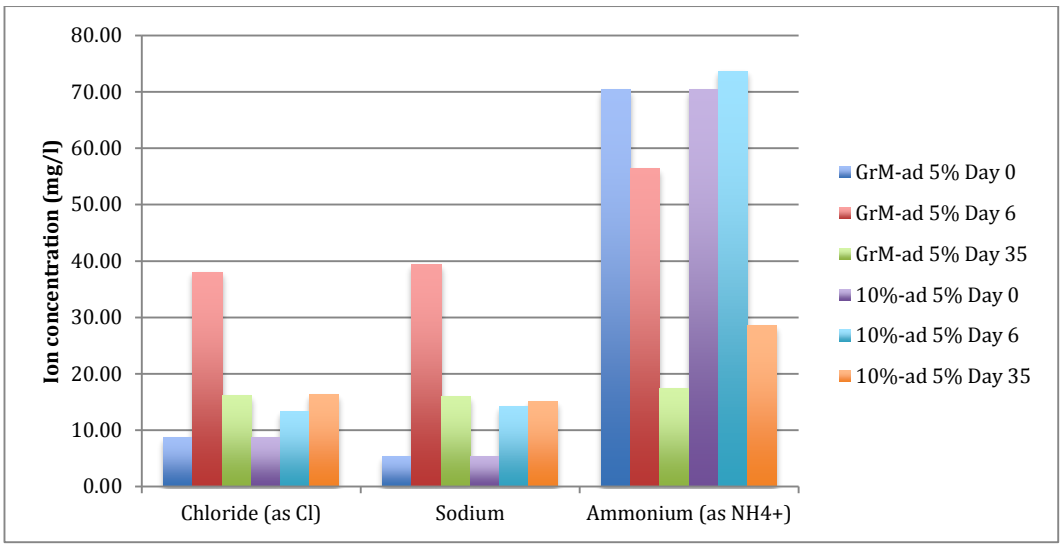

Figure 21: Mean chloride (as C), sodium and ammonium (as NH_4^+) ion concentrations of 5 % ADL solutions which contained *Scenedesmus obliquus*, measured on day 0, 6 and 35 of experiment 4. The *S. obliquus* used in this experiment had previously been growing in two different growth media: GrM-ad refers to *S. obliquus* previously grown in BBM while 10%-ad refers to *S. obliquus* previously grown on a 10% ADL solution before being sub-cultured into the solutions used in experiment 4. Each data point represents the mean of triplicate measurements and the standard errors are shown in Appendix D5.

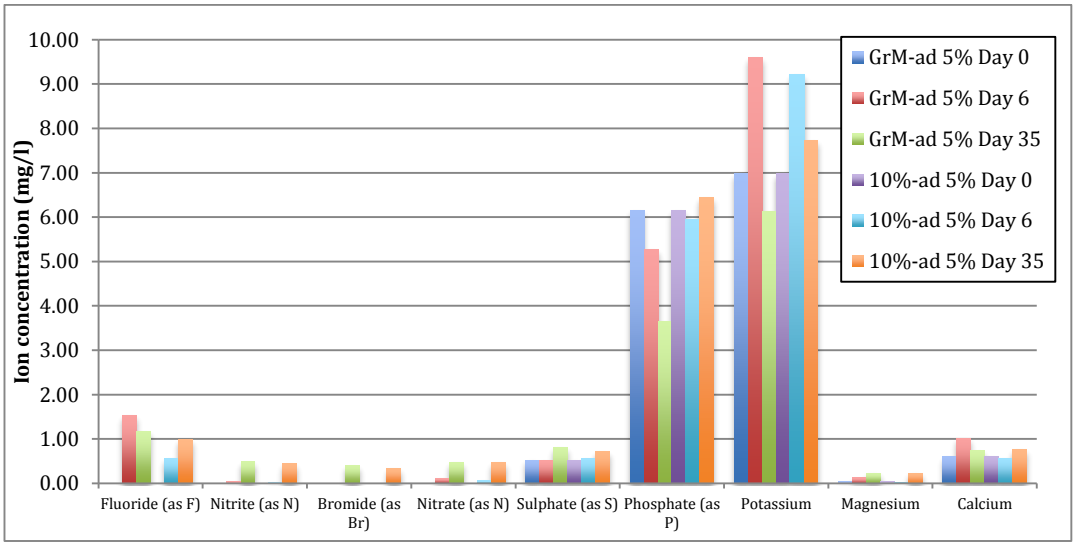

Figure 22: Mean fluoride (as F), nitrate (as N), bromide (as B), nitrate (as N), sulphate (as S), phosphate (as P), potassium, magnesium and calcium ion concentrations of 5 % ADL solutions which contained *Scenedesmus obliquus*, measured on day 0, 6 and 35 of experiment 4. The *S. obliquus* used in this experiment had previously been growing in two different growth media: GrM-ad refers to *S. obliquus* previously grown in BBM while 10%-ad refers to *S. obliquus* previously grown on a 10% ADL solution before being sub-cultured into the solutions used in experiment 4. Each data point represents the mean of triplicate measurements and the standard errors are shown in Appendix D5.

The phosphate, magnesium, potassium, fluoride, nitrate and sulphate concentration variations in 5 % ADL solutions were similar to the 1 % cultures, as shown in Figure 22. Small peaks of nitrite and

bromide were observed in both GrM-ad and 10%-ad cultures on day 35. Calcium concentrations fluctuated.

3.5.3.4 Ion chromatography results of flasks containing 10 % ADL

By comparing Figure 23, Figure 21 and Figure 19 it can be visually observed that chloride and sodium concentrations show similar temporal variation patterns in 1 %, 5 % and 10 % cultures, i.e. an increase in concentrations over the course of the experiment with an anomalous peak in the GrM-ad cultures on day 6 (see Appendix G). Ammonium is steadily depleted in the GrM-ad 10% cultures (interesting as the microalgae was in a lag phase on day 6) whilst being depleted at a much slower rate in 10%-ad 10% cultures.

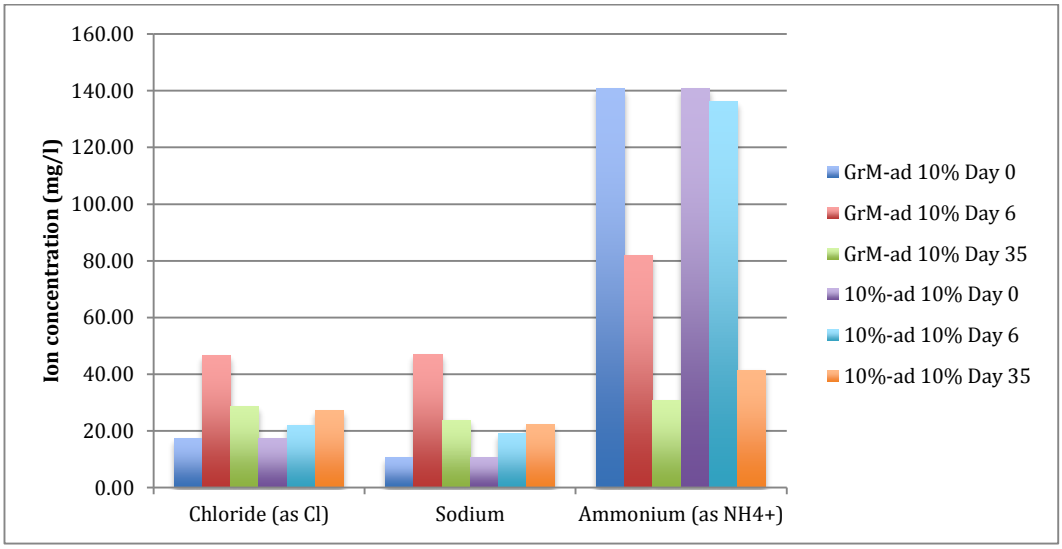

Figure 23: Mean chloride (as C), sodium and ammonium (as NH_4^+) ion concentrations of 10 % ADL solutions which contained *Scenedesmus obliquus*, measured on day 0, 6 and 35 of experiment 4. The *S. obliquus* used in this experiment had previously been growing in two different growth media: GrM-ad refers to *S. obliquus* previously grown in BBM while 10%-ad refers to *S. obliquus* previously grown on a 10% ADL solution before being sub-cultured into the solutions used in experiment 4. Each data point represents the mean of triplicate measurements and the standard errors are shown in Appendix D5.

Phosphate, fluoride and potassium concentrations in 10 % ADL solutions varied in a similar manner to 1 % and 5 % solutions, which can be seen when Figure 24 is compared to Figure 20 and Figure 22. Nitrite, nitrate and concentrations steadily increased in all flasks, as did magnesium concentrations in GrM-ad 10% flasks. Magnesium concentrations in 10%-ad cultures decreased by only 0.02 mg/l between day 1 and 6, then increase by 0.3 on day 35. Bromide concentrations showed similar patterns to 5 % cultures. Sulphate and calcium concentrations fluctuated.

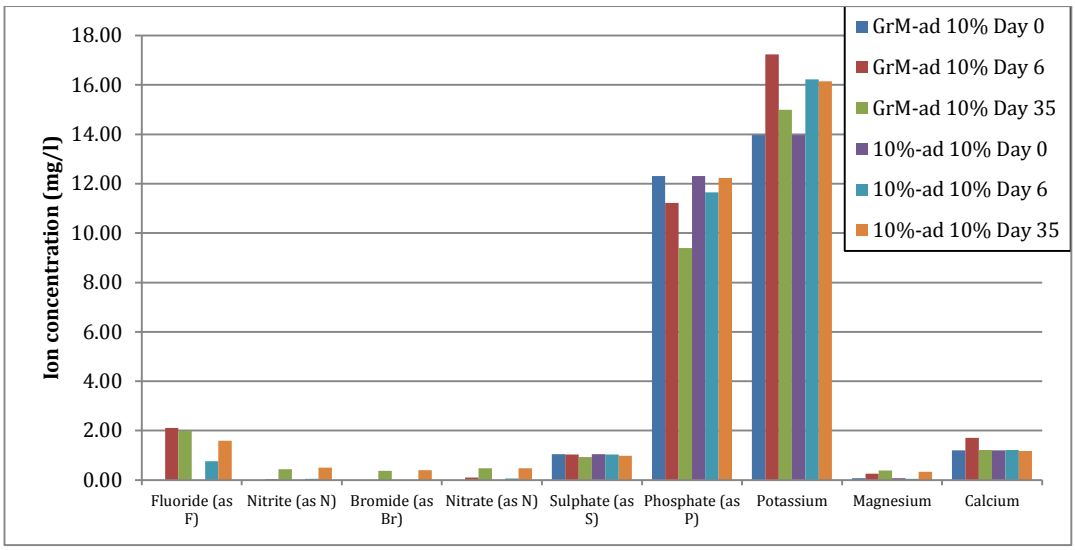

Figure 24: Mean fluoride (as F), nitrate (as N), bromide (as B), nitrate (as N), sulphate (as S), phosphate (as P), potassium, magnesium and calcium ion concentrations of 10 % ADL solutions which contained *Scenedesmus obliquus*, measured on day 0, 6 and 35 of experiment 4. The *S. obliquus* used in this experiment had previously been growing in two different growth media: GrM-ad refers to *S. obliquus* previously grown in BBM while 10%-ad refers to *S. obliquus* previously grown on a 10% ADL solution before being sub-cultured into the solutions used in experiment 4. Each data point represents the mean of triplicate measurements and the standard errors are shown in Appendix D5.

When comparing Figures 19-24 to Figure 9, it can be seen that the ion values in 1%, 5 % and 10 % concentrations are at expected levels given their dilution factor, showing that the dilution process was accurate.

3.6 Experiment 5 - Agar Contamination Check

No bacterial colonies (or microalgae) grew on any of the LB plates, showing that the cultures were not contaminated with bacteria, or if there were bacteria they were not viable for culture, as shown by Table 7. Only microalgae grew on the BBM plates. Additionally no bacterial colonies were observed on the streak and spread plates (made with BBM agar) on which *S. obliquus* was cultured, which were kept in the growth room at 25 °C.

Table 7: Contamination checks of various cultures used in different experiments (Culture column) using 1 ml of culture on spread plates (LB agar and BBM agar). Sterility of contamination check process was assessed by creating blanks, both with and without contact with the sterile glass spreader (Blank and Blank scraped plate). Cultures 10% B, GrM, JM2, 10% (27/03) and GM1 (27/03) contained *S. obliquus*. Plates were incubated at 37 °C and periodically checked between hour 0 and 144. LB = Lysogeny broth; BBM = Bold's Basal Medium.

Culture	Agar Media	Replicates
10% B	LB	2
GrM	LB	2
JM2	LB	3
Blank	LB	3
Blank scraped plate	LB	2
BBM	BBM	1
10% (27/03)	BBM	1
AD17031	BBM	1
GM1 (27/03)	BBM	1

3.7 Experiment 6 – 10 % Monitoring

3.7.1 Optical Density measurements

Figure 25 shows that ODs decreased in all cultures until after day 5 and most cultures did not enter exponential growth until after day 7 (Figure 62, Appendix F). The highest total biomass and growth rate occurred in the 10%-ad 10% A culture (0.158 d^{-1} on day 21), however, overall there does not appear to be a very distinct difference between 10%-ad cultures and GrM-ad cultures.

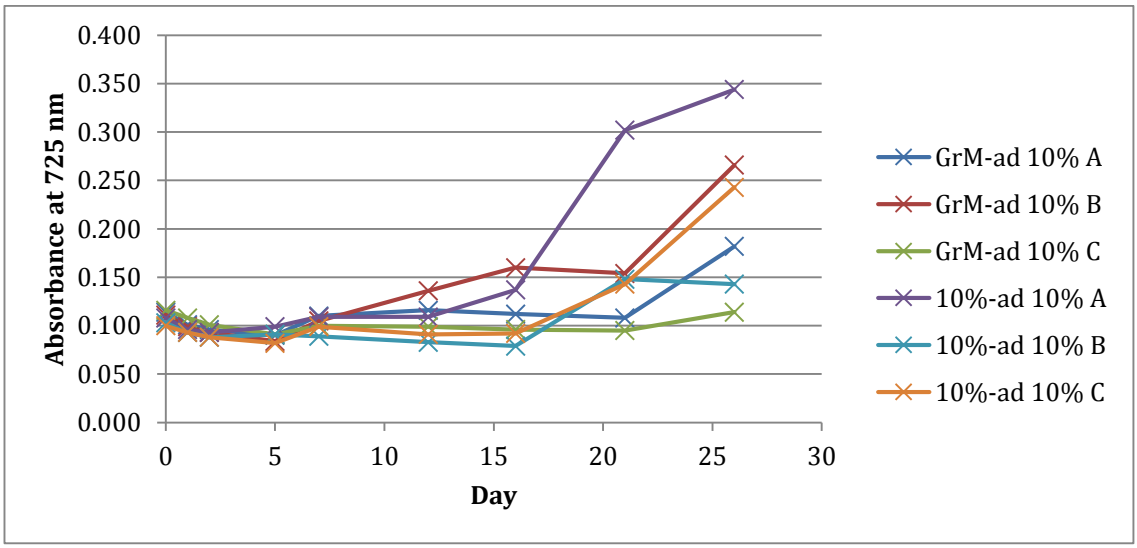

Figure 25: OD measurements of *Scenedesmus obliquus* previously grown on BBM (GrM-ad) and 10 % ADL (10%-ad) solutions, sub-cultured into 10 % ADL solutions (diluted with DI water).

3.7.2 pH measurements

pH dropped in all cultures over time, as shown in Figure 26. The fastest decrease was between day 0 and 2, with the cultures dropping from pH 9.30 to pH 8.87-8.95. However pH continued to decrease in all cultures over the 26 day period and when compared to Figure 25, there appears to be a loose correlation between growth and pH decrease.

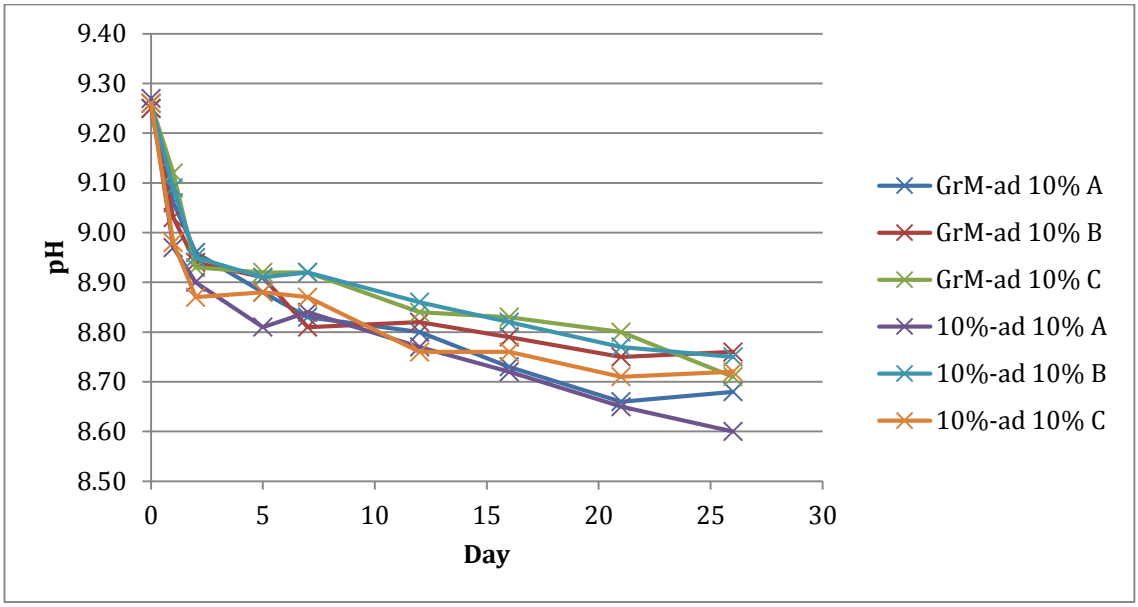

Figure 26: pH measurements of *Scenedesmus obliquus* previously grown on BBM (GrM-ad) and 10 % ADL (10%-ad) solutions, sub-cultured into 10 % ADL solutions (diluted with DI water).

3.7.3 Optical Density vs. pH

Figure 27 shows OD (or growth) against pH. It can be seen that no growth occurs at pHs greater than 8.90. Below this pH value the general trend is that growth increases as pH decreases. Calculated ammonia concentrations at various pH levels are also shown. The window where growth begins is similar to the threshold values reported in the literature by Collos and Harrison (2014) and Bjornsson et al. (2013) 17.62 – 23.0 mg/l NH_3-N. The calculated NH_3-N values are not perfect as they assume unchanging total ammonia nitrogen (TAN) concentrations throughout growth. However it is reasonable to assume that little ammonia is consumed during a lag phase, so until exponential growth has occurred, the calculated NH_3-N line is considered to be acceptable by the author.

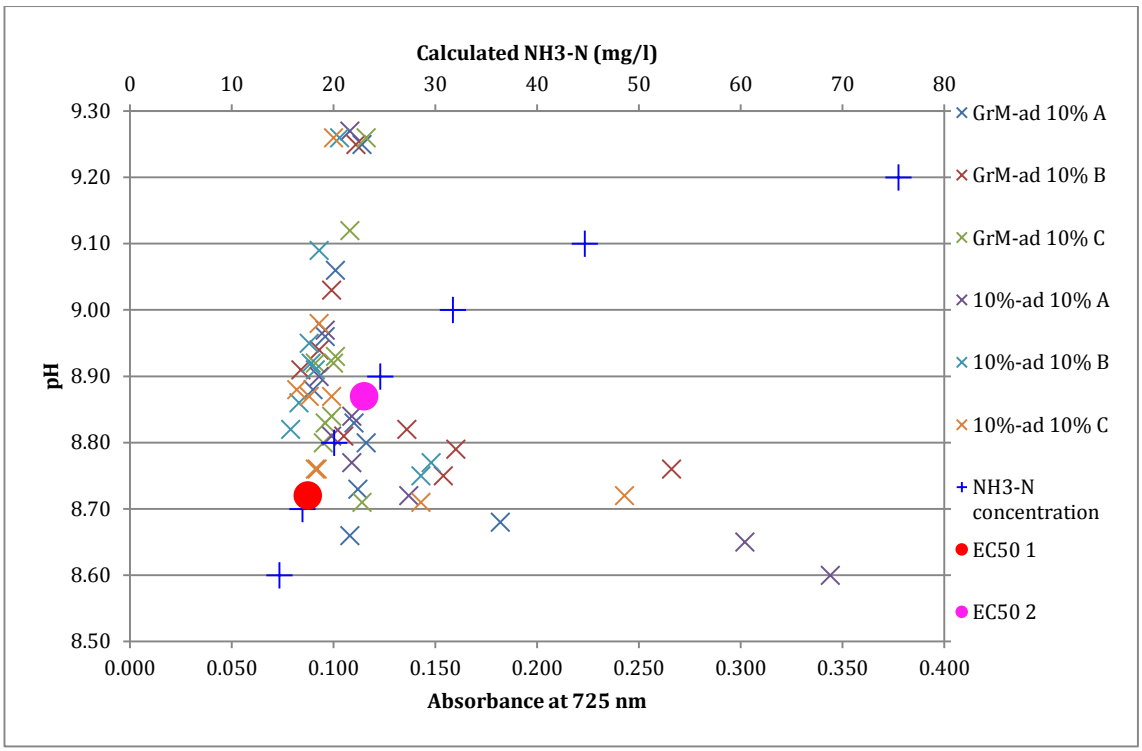

Figure 27: OD measurements taken in Experiment 6 plotted against pH measurements taken in Experiment 6. The calculated NH_3-N concentration is also shown against pH values 8.60-9.20. EC50 1 and EC50 2 refer to threshold ammonia concentrations mentioned in the literature. Collos and Harrison (2014) calculate that the EC_{50} of NH_3-N in *S. obliquus* is 17.62 mg/l, and EC50 1 is plotted at 17.50 mg/l NH_3-N. Bjornsson et al. (2013) state that *S. obliquus* can grow at NH_3-N concentrations lower than 23 mg/l NH_3-N; EC50 2 is plotted at 23.02 mg/l NH_3-N.

3.8 Experiment 7 – Microscopy

There were only 2 bacteria visible in the GrM-ad GrM A culture sample (which may have been introduced by the microscopy process) and the microalgae cells were oval, green and all looked relatively similar. By contrast, the GrM-ad 10% B culture contained a significant number of bacteria (the specific number was not counted). The microalgae cells were spherical and most of them were small and transparent. There were a few large spherical cells that were very green. Additionally, a lot of unidentifiable debris was observed littering the culture – it may have been digestate, but it seemed likely that it was the remains of dead cells. There were no cameras available capable of photographing the cultures at a magnification greater than ×100.

3.9 Experiment 8 - Buffer and Trace Elements Test

3.9.1 Optical density measurements

Unfortunately the microalgae in the 10%-ad flasks flocculated into such large clusters that it was not possible to measure the OD accurately. This was likely due to the high levels of contamination in the parent culture (see Figure 33c). Unfortunately the microalgae in the 10%-ad flasks flocculated into such large clusters that it was not possible to measure the OD accurately. This was likely due to the high levels of contamination in the parent culture.

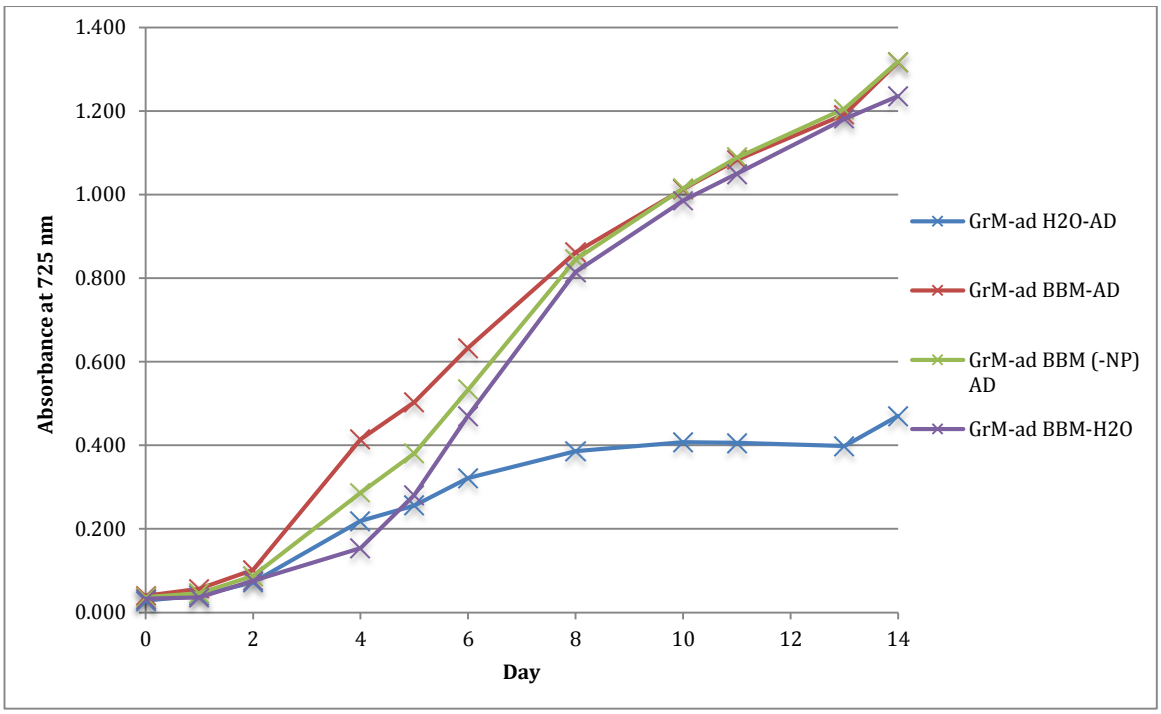

Figure 28: Mean OD measurements of *Scenedesmus obliquus* previously grown on BBM (GrM-ad) growing in buffered BBM and 10 % ADL solutions (with and without trace element supplementation). The flasks contained growth medium diluted with 10 % DI water (BBM-H_2O) or 10 % ADL diluted with 90 % DI water (H_2O-AD), growth medium (BBM-AD) or modified growth medium (BBM (-NP) AD). Each data point represents the mean of triplicate measurements and standard errors are shown in Appendix D6.

3.9.2 pH measurements

It can be seen from Figure 29 that there was little difference in pH change between GrM-ad and 10%-ad cultures. Despite being in a buffered solution, the pH still changed over the course of the experiment. pH increased in growth medium solutions, from 6.90 at the beginning of the experiment to just over 7.20 by the end. pH in the flasks containing ADL all followed a similar patter; increasing from approximately 7.60 to 7.80 over the first 21.75 hours, plateauing at this level until hour 94.75 (although no pH measurement was taken in between, so there is a chance that the pH could have continued to rise and then fallen again), then decreased steadily until hour 334.

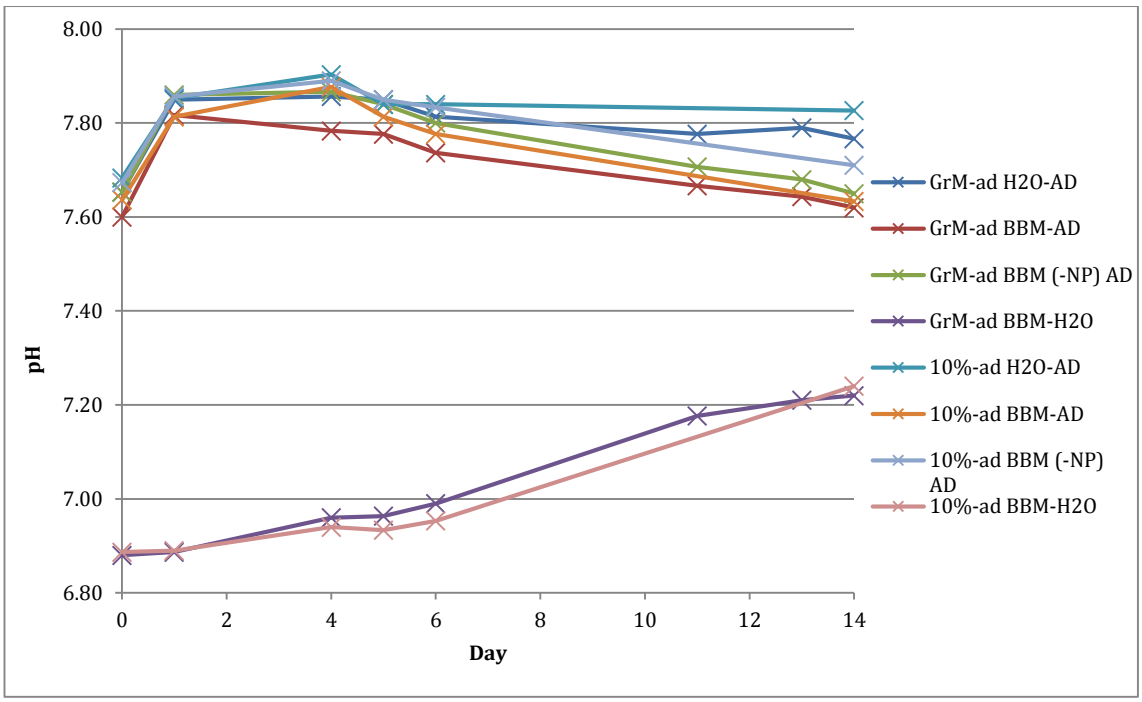

Figure 29: Mean pH measurements of solutions containing *Scenedesmus obliquus* previously grown on BBM (GrM-ad) and 10 % ADL (10%-ad). The *S. obliquus* was sub-cultured into buffered growth medium and 10 % ADL solutions (with and without trace element supplementation). The flasks contained growth medium diluted with 10 % DI water (BBM-H_2O) or 10 % ADL diluted with 90 % DI water (H_2O-AD), growth medium (BBM-AD) or modified growth medium (BBM (-NP) AD). Each data point re.presents the mean of triplicate measurements and standard errors are shown in Appendix D6.

3.9.3 Ion chromatography measurements

By observing Figure 30 it can be observed that fluoride concentrations increased over the fortnight. Nitrite increased from practically 0 to approximately 0.5 mg/l (except in GrM-ad BBM (-NP) AD, GrM-ad BBM and 10%-ad BBM where no nitrite was recorded on the first or 14th days). Bromide decreased in all cultures that contain ADL and no bromide was recorded in pure BBM cultures. In H_2O-AD cultures the magnesium concentration increased over the fortnight. In the rest of the cultures the magnesium concentration reduced over the fortnight. In the all the cultures except for 10%-ad BBM-AD, the calcium concentration increases over the fortnight.

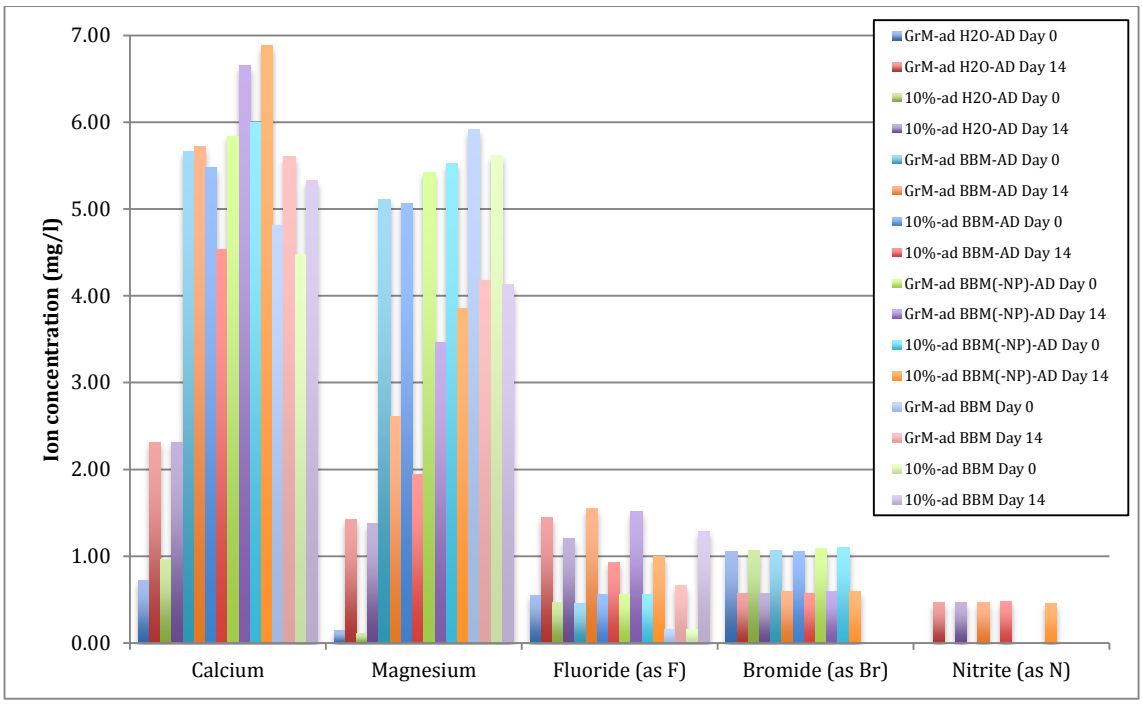

Figure 30: Mean IC measurements of calcium, magnesium, fluoride (as F), bromide (as Br) and nitrite (as N) present in solutions containing *Scenedesmus obliquus,* previously grown on BBM (GrM-ad) and 10 % ADL (10%-ad). The *S. obliquus* was sub-cultured into buffered growth medium and 10 % ADL solutions (with and without trace element supplementation). The flasks contained growth medium diluted with 10 % DI water (BBM-H_2O) or 10 % ADL diluted with 90 % DI water (H_2O-AD), growth medium (BBM-AD) or modified growth medium (BBM (-NP) AD). Each data point represents the mean of triplicate measurements and standard errors are shown in Appendix D6. Measurements were taken on day 0 and day 14 of the experiment.

Nitrate concentrations essentially remained the same in all the cultures that contained ADL, as shown in Figure 31. In the cultures containing pure growth medium, the nitrate concentrations dropped significantly over the fortnight. This shows that ammonium was preferentially consumed over nitrate when available. The sulphate concentrations decreased in all cultures. A small reduction in phosphate concentrations was observed in all cultures. The potassium concentrations decreased slightly in all cultures except for GrM-ad H_2O-AD and GrM-ad BBM, where slight increases were observed.

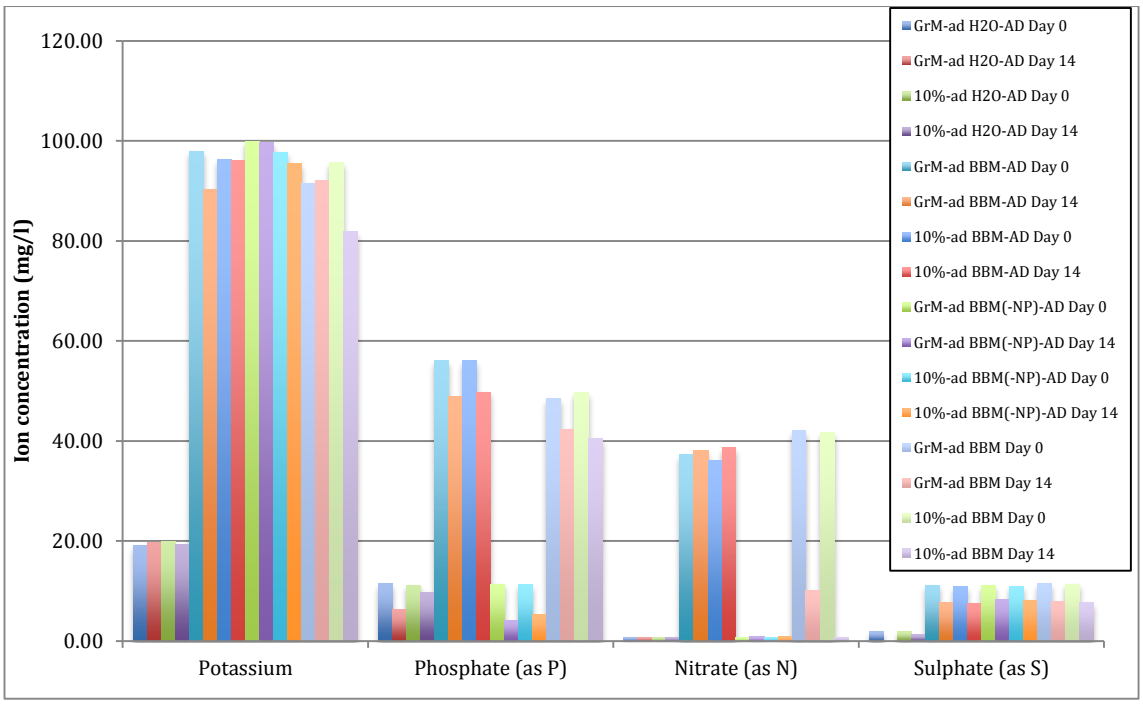

Figure 31: Mean IC measurements of potassium, phosphate (as P), nitrate (as N) and sulphate (as S) ions present in solutions containing *Scenedesmus obliquus,* previously grown on BBM (GrM-ad) and 10 % ADL (10%-ad). The *S. obliquus* was sub-cultured into buffered growth medium and 10 % ADL solutions (with and without trace element supplementation). The flasks contained growth medium diluted with 10 % DI water (BBM-H_2O) or 10 % ADL diluted with 90 % DI water (H_2O-AD), growth medium (BBM-AD) or modified growth medium (BBM (-NP) AD). Each data point represents the mean of triplicate measurements and standard errors are shown in Appendix D6. Measurements were taken on day 0 and day 14 of the experiment.

By observing Figure 32 it can be seen that the sodium and chloride concentrations remained similar throughout the experiment (unsurprising as they are mainly biologically inert). The ammonium concentrations decreased in all flasks except for the pure BBM cultures where no ammonium was present. The smallest decrease in ammonium concentrations was seen in 10%-ad H_2O-AD. This may have been due to reduced microalgae growth and uptake, in turn due to the bacterial contamination. However, despite much lower overall growth and a greater presence of bacteria, a similar amount of ammonium was removed from GrM-ad H_2O-AD as from GrM-ad BBM-AD and GrM-ad BBM (-NP) AD.

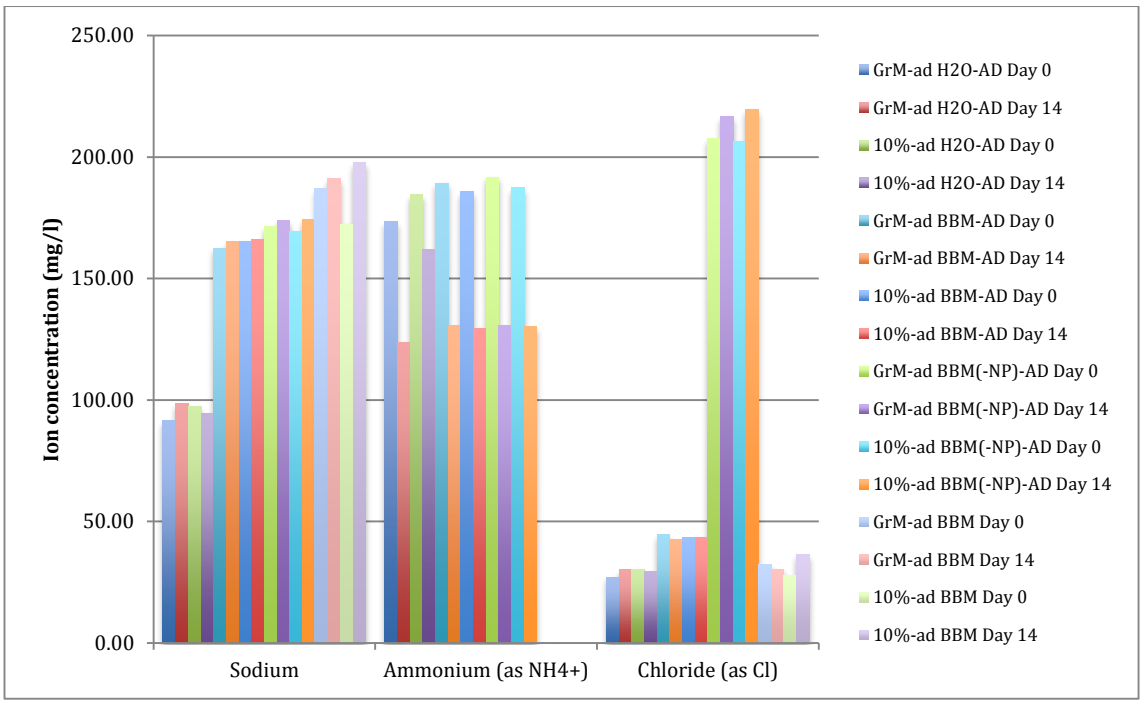

Figure 32: Mean IC measurements of sodium, ammonium (as NH_4^+) and chloride (as Cl) ions present in solutions containing *Scenedesmus obliquus,* previously grown on BBM (GrM-ad) and 10 % ADL (10%-ad). The *S. obliquus* was sub-cultured into buffered growth medium and 10 % ADL solutions (with and without trace element supplementation). The flasks contained growth medium diluted with 10 % DI water (BBM-H_2O) or 10 % ADL diluted with 90 % DI water (H_2O-AD), growth medium (BBM-AD) or modified growth medium (BBM (-NP) AD). Each data point represents the mean of triplicate measurements and standard errors are shown in Appendix D6. Measurements were taken on day 0 and day 14 of the experiment.

3.9.4 Transmission Electron Microscopy images

TEM images were taken of 5 cultures. The two parent cultures, one growth medium and the other 10 % ADL. The three daughter cultures analysed were GrM-ad H_2O-AD, GrM-ad BBM (-NP) AD and GrM-ad BBM-H_2O.

3.9.5.1 10 % ADL:

Figure 33a, Figure 33b and Figure 33c show that there is a great deal of bacterial contamination in the parent 10 % culture, although the bacteria only represent 1 % of the biomass (this was calculated by analyzing Figure 33b, assuming all cells were circular, measuring the longest axis of each cell including broken or transparent cells but excluding cells which were only partially on the image). There are a lot of dead, lysed cells and also a number of (what looks like) transparent cells. The larger cells tend to be 4-5 μm and cell shapes and cell edges are irregular.

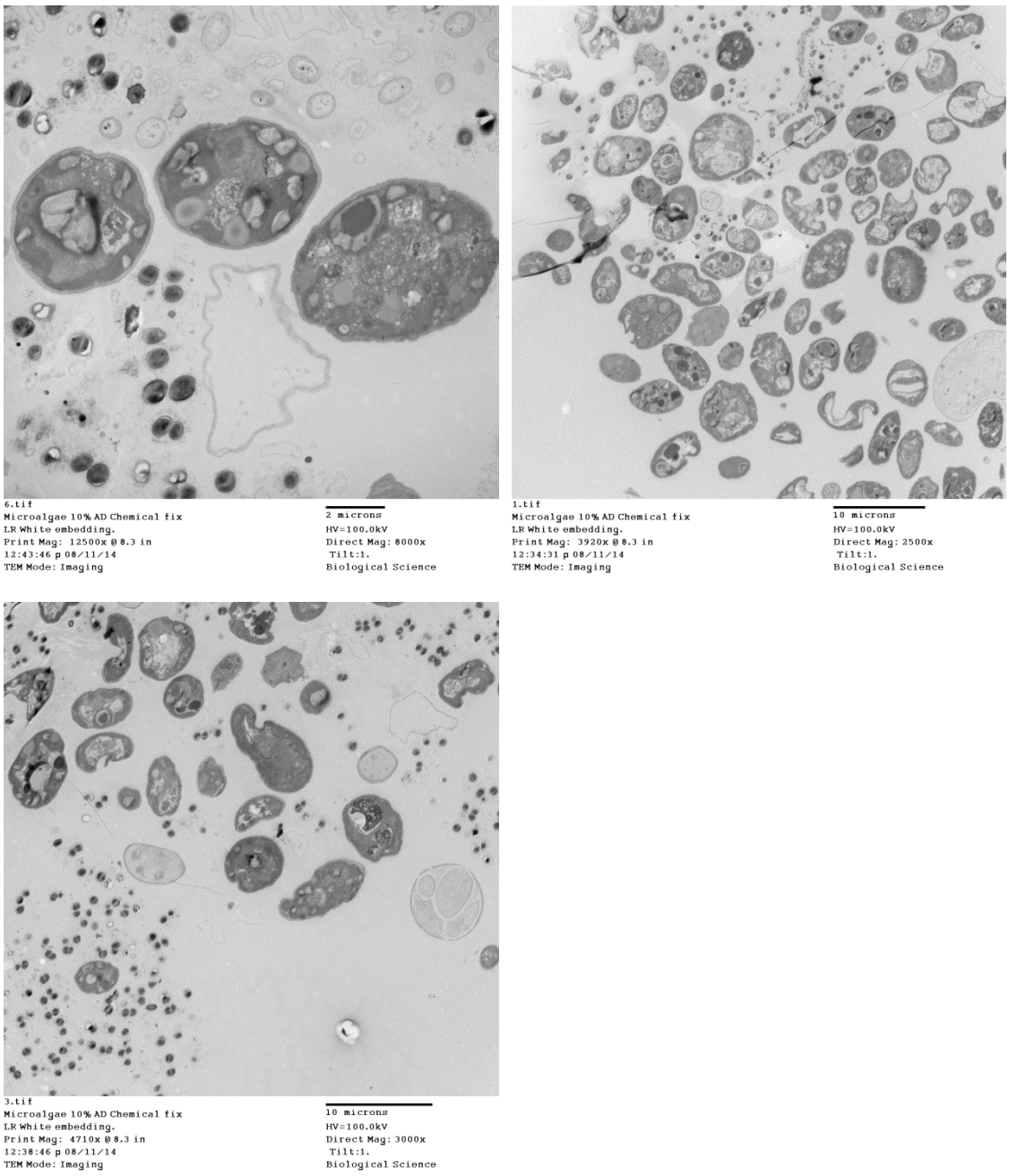

Figure 33a, b & c: TEM images of *Scenedesmus obliquus* cells grown in a 10 % ADL solution. This culture was used as a parent culture in Experiment 8. The three images show the same culture, with (a) showing a close up of three *S. obliquus* cells, while (b) and (c) show a less magnified view displaying the variety of cell shapes and bacterial contamination present.

3.9.5.2 BBM

There is minimal bacterial contamination in the BBM parent culture shown in Figure 34a and Figure 34b, despite having been incubated for the same period of time as the microalgae shown in Figure 33a, Figure 33b and Figure 33c. The cells are a similar size to those in the 10 % A flask, however they are oval shaped and have smooth, very thick cell walls. They appear to have a lot of lipid storage, presumably due to nitrogen limitation.

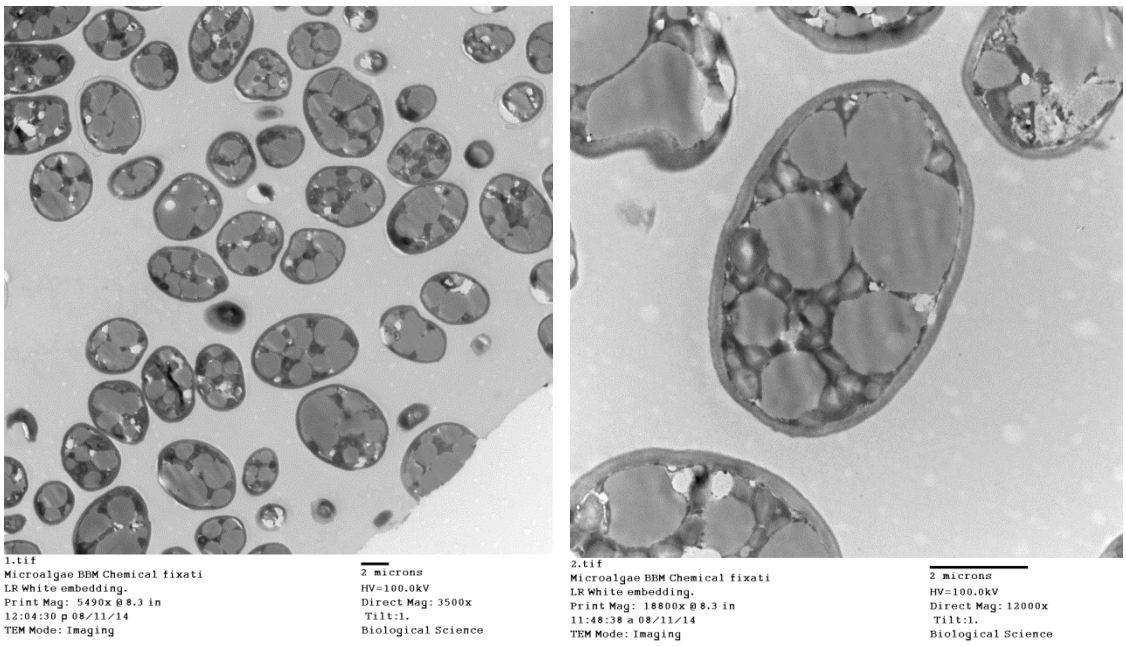

Figure 34a & b: TEM images of *Scenedesmus obliquus* cells grown in BBM. This culture was used as a parent culture in Experiment 8. (a) The culture contains multiple, relatively uniform *S. obliquus* cells, few broken cells and no bacterial contamination; (b) magnified view of a single *S. obliquus* cell shows a thick cell wall and large lipid deposits.

3.9.5.3 GrM-ad H_2O-AD

Figure 35a and Figure 35b show microalgae from the parent BBM culture (shown in Figure 34a and Figure 34b) sub-cultured into a 10 % ADL solution diluted with DI water (buffered to pH 7). Figure 35a shows that bacteria quickly proliferated in this culture after 14 days, by which point 5 % of the biomass was made up of bacteria, even though the bacteria levels in the parent culture were low after two months of incubation. The figure of 5 % was calculated by measuring the longest axis of all of the microalgae and bacteria cells in Figure 35 a (including broken cells but excluding cells only partially shown by the TEM image), and assuming all cells were circular. The cell density is low in comparison to the other cultures tested with TEM and the cells have irregular shapes, but tend to be more round than oval. Figure 35b shows the internal structure of one of the cells. The chloroplast is very thin and takes up a small proportion of the cell. It also appears to have large storage granules – potentially starch.

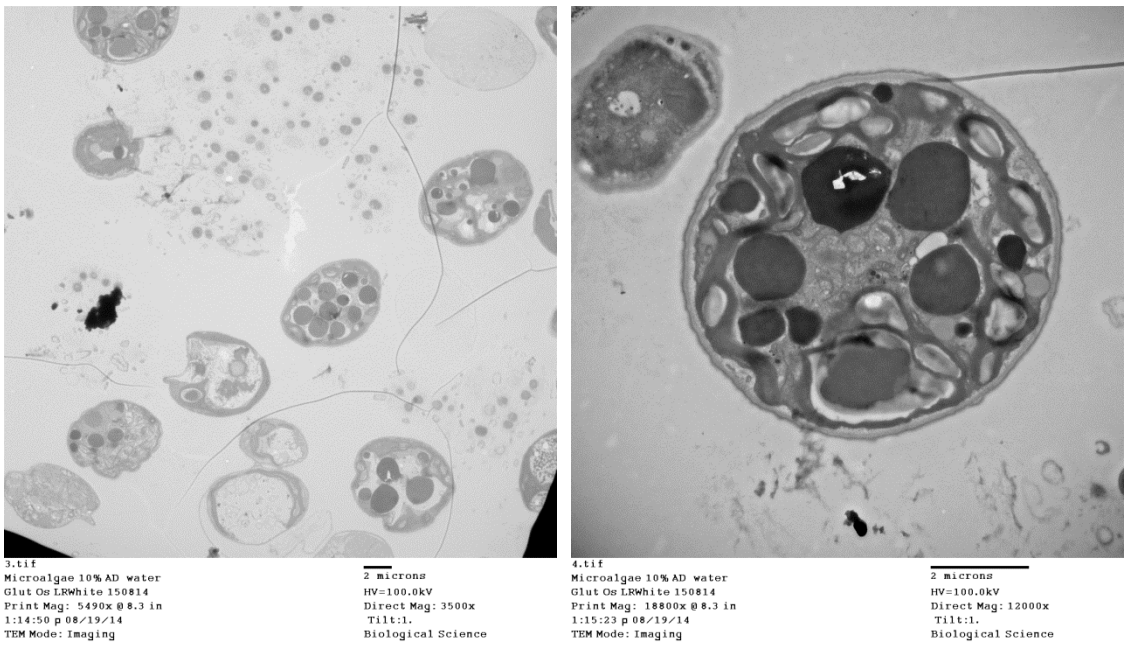

Figure 35a & b: TEM images of *Scenedesmus obliquus* cells in the GrM-ad H_2O-AD culture after 14 days. (a) *S. obliquus* cells are large and circular and the culture is heavily contaminated with bacteria; (b) magnified view of a single *S. obliquus* cell shows a thin chloroplast and potential starch storage granules.

3.9.5.4 GrM-ad BBM (-NP) AD

Figure 36a and Figure 36b show microalgae from the parent BBM culture (shown in Figure 34a and Figure 34b) sub-cultured into a solution of 10 % ADL diluted with modified growth medium (without any nitrate or phosphate), buffered to pH 7.

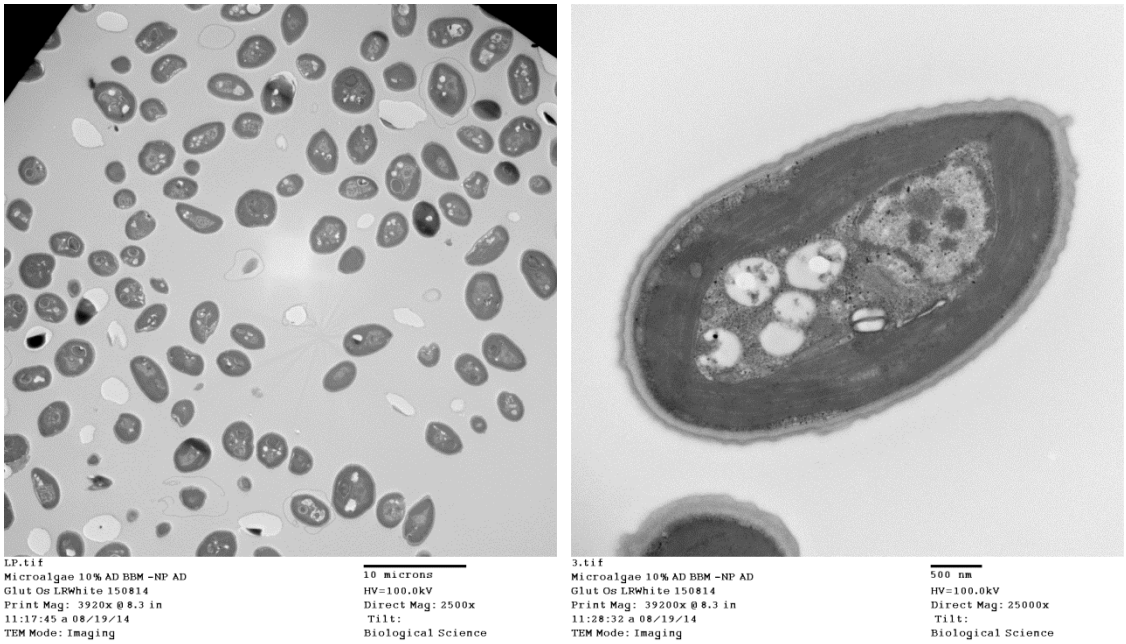

Figure 36a & b: TEM images of *Scenedesmus obliquus* cells, previously grown in BBM and sub-cultured into BBM (-NP) AD for 14 days. (a) No visible evidence of bacterial contamination or cell debris. Most cells are similar sizes and tear-drop shaped; (b) magnified view of a single *S. obliquus* cell shows a large, single chloroplast.

Compared to GrM-ad H_2O-AD (Figure 35a), there is very little bacterial contamination in the GrM-ad BBM (-NP) AD flask. The cells are teardrop shaped, have smooth edges and contain a single very large chloroplast (Figure 36b).

3.9.5.5 GrM-ad BBM H_2O

Figure 37a and Figure 37b show microalgae from the parent BBM culture (shown in Figure 34a and Figure 34b) sub-cultured into growth medium (buffered to pH 7). The appearance of the cells is similar to those grown in GrM-ad BBM (-NP) AD in Figure 36a and Figure 36b. The cells are teardrop shaped, have a large chloroplast and there is little bacterial contamination in the culture.

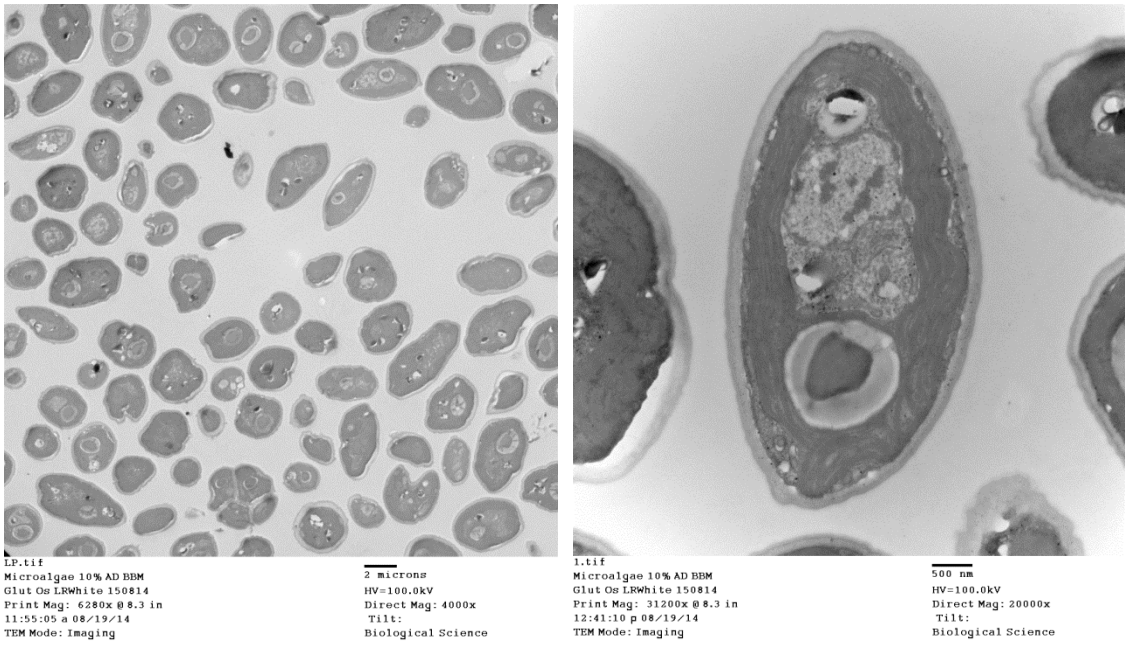

Figure 37a & b: TEM images of *Scenedesmus obliquus* cells, previously grown in BBM and sub-cultured into BBM H_2O (90 % Bold's Basal Medium, 10 % DI water) for 14 days. (a) No visible evidence of bacterial contamination or cell debris. Most cells are similar sizes and tear-drop shaped; (b) magnified view of a single *S. obliquus* cell shows a large, single chloroplast – similar to those seen in BBM (-NP) AD culture.

3.9.5 Photos of colour change in flasks

Microalgae grown in flasks containing digestate and growth medium appeared to have a much more yellowy green colour than the microalgae grown in growth medium, this can be observed in Figure 38a. However this was not the case in GrM-ad BBM (-NP) AD. This had the same dark green colour as the microalgae grown in digestate, as did GrM-ad BBM-AD (Figure 38b). It can be seen from Figure 38c that the colour difference is in the microalgae cells and is not due to water colour. It is not possible to prove from these photos that the colour difference is not due to cell density, however the author observed that no matter the stage of growth or cell density, cultures grown in BBM always had a dark green colour until they were about 2 months old, while cultures grown in 10 % ADL always had a lighter yellowy-green colour. Figure 38d shows the extent of the flocculation in the 10 %-adapted flasks, demonstrating why OD measurements were not possible.

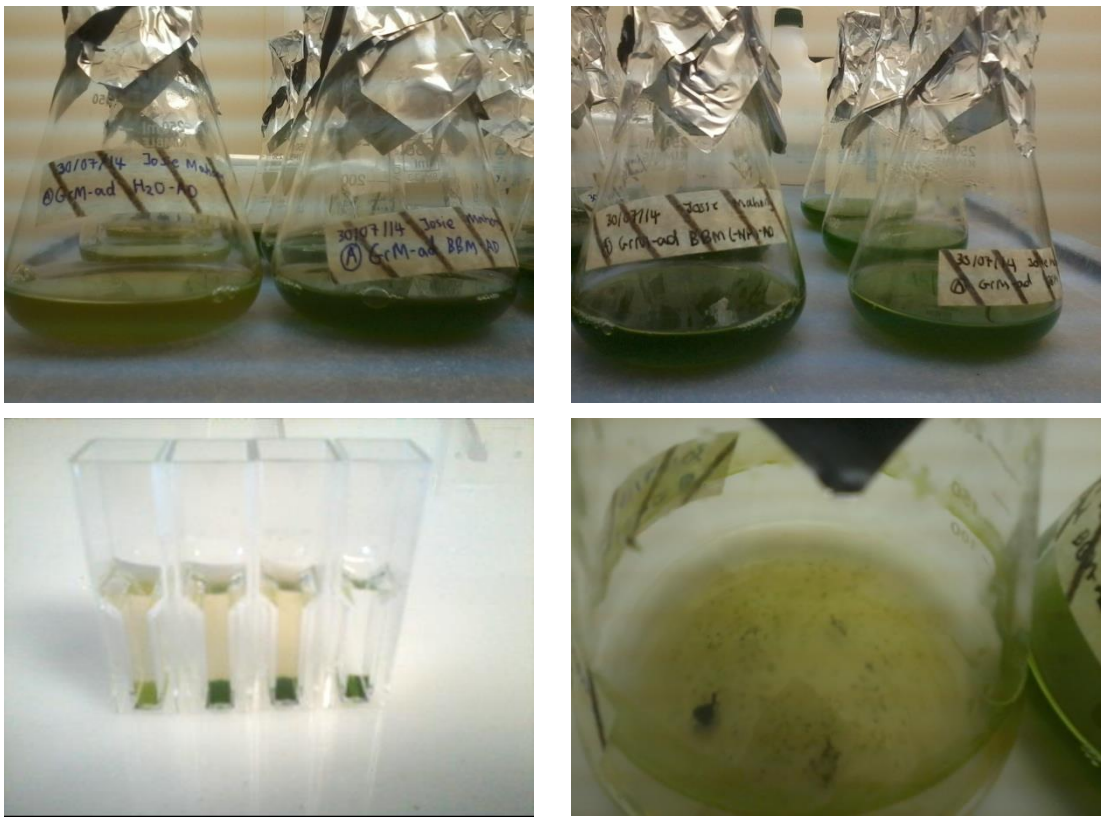

Figure 38a, b, c & d: Photos of colour changes in flasks. Figures 39a and 39b (top left and right respectively) show the colour difference between the GrM-ad H_2O-AD flask and GrM-ad BBM-AD flasks, and the GrM-ad H_2O-AD flask and GrM-ad BBM-AD flasks respectively. Figure 39c (bottom left) shows colour change of settled cells in cuvettes. From the left the cultures are GrM-ad H_2O-AD, GrM-ad BBM-AD, GrM-ad BBM (-NP) AD and GrM-ad BBM-H_2O. This exposure of this photo was increased to better illustrate the colour changes – the unedited photo can be seen in Appendix B9. Figure 39d (bottom right) shows the flocculation that occurred in flasks pre-adapted to 10 % ADL (this culture was a 10%-ad H_2O-AD flask. Similar flocculation occurred in all 10%-ad flasks).

3.10 Experiment 9 - Unsterile Culturing Experiment

3.10.1 Optical Density measurements

No growth occurred in any of the flasks in the first 54 hours, a fact demonstrated in Figure 39. After this point no more OD measurements were taken, due to flocculation, no visible growth (green tinge/rim in flasks) and a knowledge that the pH was too high for the microalgae to be able to grow in (due to the pH driving up free ammonia concentrations – see Section 4.2.3).

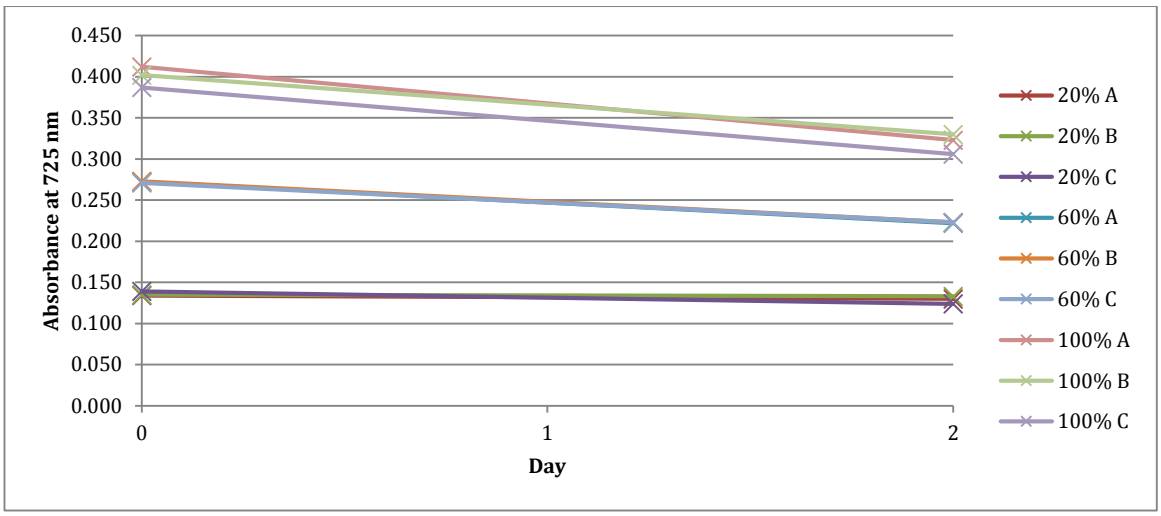

Figure 39: OD measurements of *Scenedesmus obliquus* grown in 20 %, 60 % and 100 % ADL solutions (diluted with tap water).

In this experiment, the greater the concentration of ADL, the greater the decrease in OD (implying that the higher the concentration of OD, the greater the cell death) and this is shown in Figure 40. However there must be other reasons for the decrease in OD, such as changing properties in the OD, or simply error in measurement as the 100 % cultures show a negative OD when the initial OD of the ADL is removed.

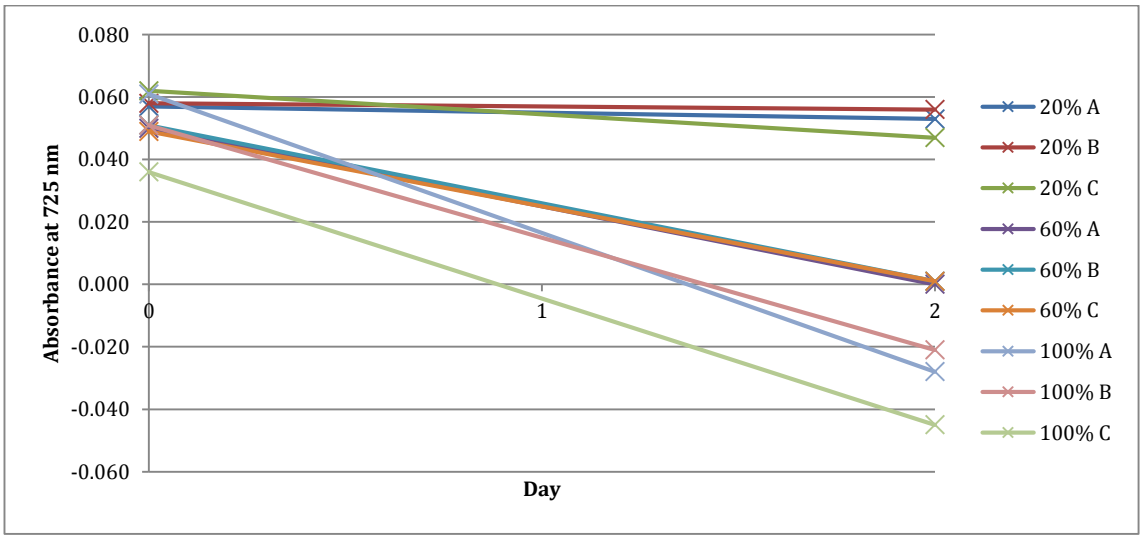

Figure 40: Normalised OD measurements (OD of digestate removed) of *Scenedesmus obliquus* grown in 20 %, 60 % and 100 % ADL solutions (diluted with tap water).

3.10.2 pH measurements

pH fluctuations over the course of the experiment are shown in Figure 41. Hour 0-2.5 shows the pH decrease due to the addition of dry ice. However the pH rapidly returned to the previous high levels. At hour 144.25 the pH was decreased by dissolving $NaHCO_3$ (it was theorized that dry ice would damage the dormant microalgae). The next day the pH had increased again and the experiment was ended, as it was unlikely that microalgae would be able to grow in such high ammonium concentrations, at such a high pH.

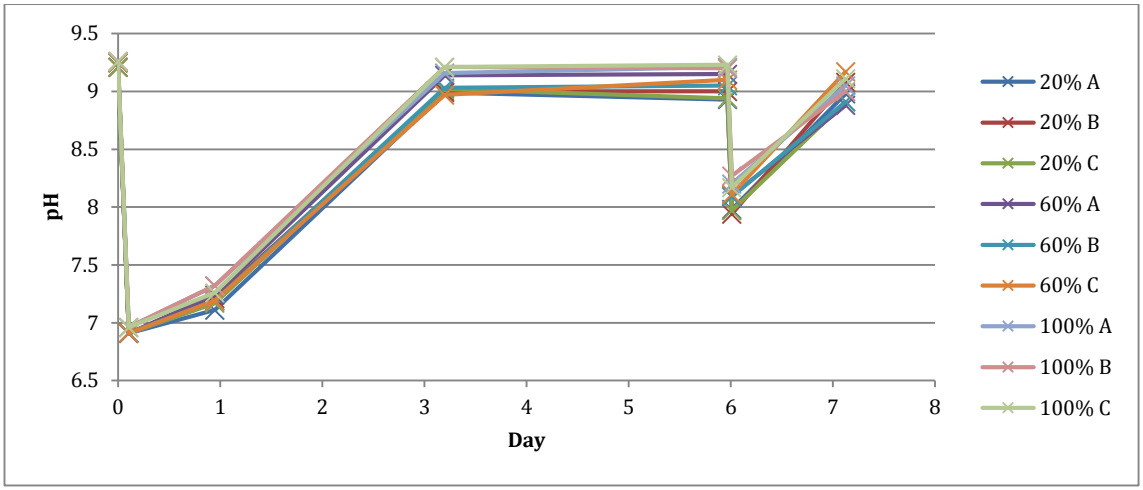

Figure 41: pH measurements of *Scenedesmus obliquus* grown in 20 %, 60 % and 100 % ADL solutions (diluted with tap water).

3.11 Growth rate in growth medium

The fastest growth rates (k) recorded in GrM-ad growth medium cultures in three experiments, between the start of the experiment and a specific day are shown in Table 8. For full calculations see Appendix C2. The highest growth rate was recorded in growth medium was 0.445 (d^{-1}) in Experiment 8. This is less than the 0.568 (d^{-1}) recorded in GrM-ad BBM (-NP) AD a (Table 9).

Table 8: Fastest mean growth rate (k) values in growth medium in Experiment 1, 4 and 8. Growth rate was calculated between the start of the experiment and each day of the experiment, to gain an average growth rate over the course of the experiment.

Growth Medium Culture	Days	k (d^{-1})
Experiment 1 – GrM	1-5	0.381
Experiment 4 - GrM-ad GrM	0-5	0.356
Experiment 8 - GrM-ad BBM-H_2O	0-6	0.445

3.12 Normalized growth rates in 10 % ADL

The normalized growth curves of microalgae growing in 10 % ADL in 3 experiments (the lag and OD at the start of the lag have been removed) are shown in Figure 42. The least biomass accumulation and slowest growth is seen in the GrM-ad culture in Experiment 4 (max growth rate 0.111 d^{-1}). The maximum growth rate in "Experiment 8 GrM-ad H_2O-AD" is high at 0.512 d^{-1}, however the growth rates decrease after 120 hours and thus the amount of biomass accumulated is relatively low at 300 hours. Both "Experiment 1 10%" and "Experiment 8 GrM-ad BBM (-NP) AD" achieve a large volume of biomass in a relatively short time (approximately 3× greater than "Experiment 8 GrM-ad H_2O-AD"). However, the maximum growth rate in "Experiment 1 10%" is 0.264 d^{-1} whilst in "Experiment 8 GrM-ad BBM (-NP) AD" it is 0.512 d^{-1}. This difference is due to the cell density in Experiment 1 being higher at the end of the lag period, in comparison the Experiment 8 GrM-ad BBM (-NP) AD.

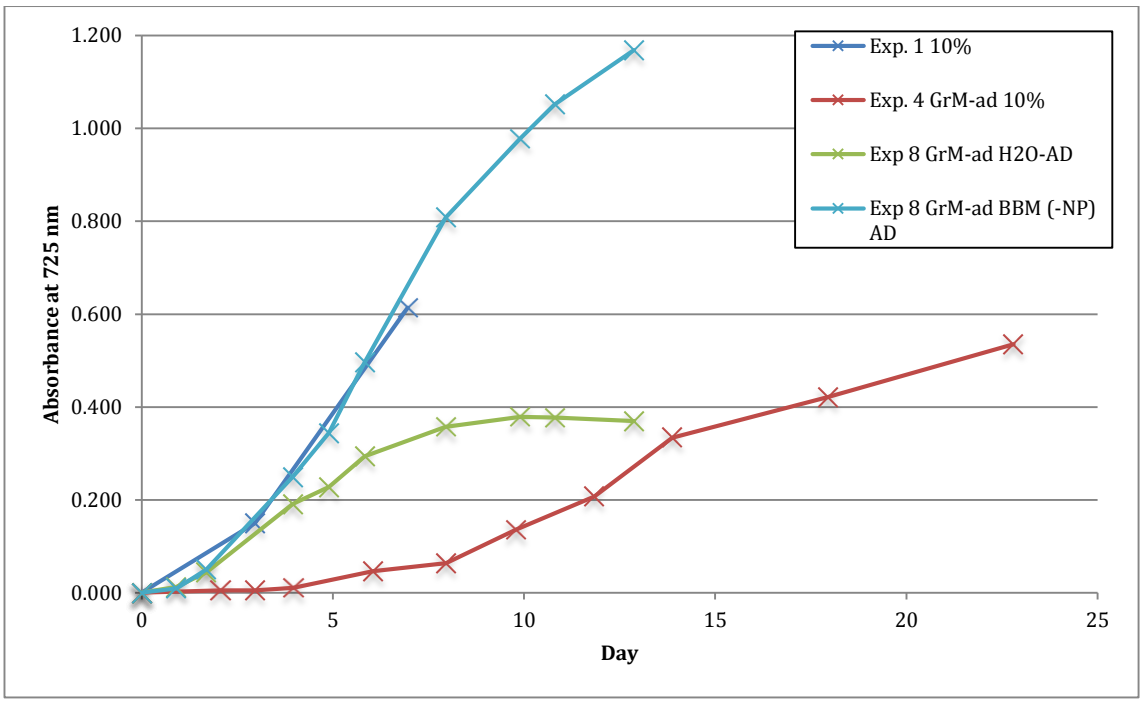

Figure 42: Comparison of growth curves in 4 flasks over 3 experiments (with lag time and OD at beginning of lag removed). Each data point is an average of triplicate measurements, standard errors shown in Appendix D.

3.13 Ammonium speciation

The speciation of ammonium between pH 7.00 and 9.00 at different dilutions is shown in Figure 43a to Figure 43e.

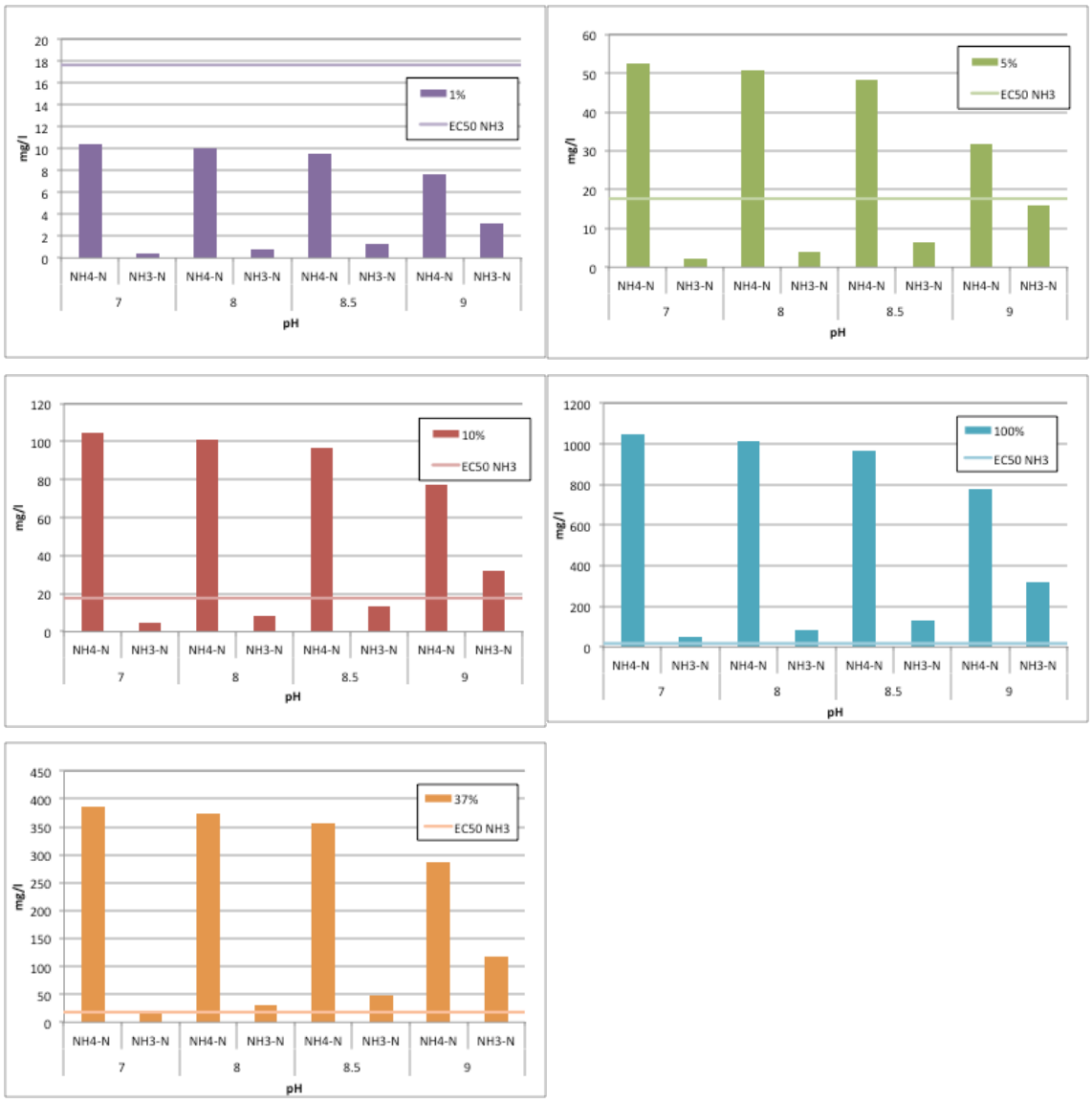

Figure 43a, b, c, d & e: Ammonium speciation at different pH values; (a) 1 % (top left), (b) 5 % (top right), (c) 10 % (middle left), (d) 100 % (middle right) and (e) 37 % (bottom left) dilutions of AD1703 (measured for Experiment 4), at pH's between 7 and 9. The NH_3-N EC_{50} was calculated for *S. obliquus* by Collos and Harrison (2014).

The value of 1093.21 mg/l TAN was used in the Figure 43 calculations. The EC_{50} value was calculated for *S. obliquus* in Collos and Harrison (2014). The NH_4^+-N: NH_3-N ratio and concentrations were calculated using the procedure outlined by FDEP (2001).

The calculated ammonium speciation in a 37 % solution of ADL is shown in Figure 43e. This is roughly 400 mg/l TAN (404.5 mg/l to be precise) and at pH 7, at this concentration, the concentration of NH_3–N is 17.25 mg/l, extremely close to the EC_{50} level of 17.62 mg/l NH_3-N. Theoretically, at pH 7.00, and at this concentration, growth would be inhibited by 50 % and this inhibition would only increase as the TAN concentration increased. This means it is unlikely that concentrations greater

than 400mg/l TAN can be remediated rapidly, which is unfortunate given that the concentrations of ammonium in unautoclaved ADL are typically 1500 mg/l TAN (see Table 15 - Appendix A2).

3.14 Nutrient calculations

Table 9 shows estimates of how long would be required for microalgae to strip 400 mg/l TAN from digestate, based on growth rates recorded in Experiment 8. Further details of the calculations used to create Table 9 can be found in Appendix C1. 400 ml TAN was chosen as a remediation target as it is approximately the EC_{50} value of ammonia at pH 7 (Figure 43e and Collos and Harrison (2014)). The calculation assumes an initial OD (a_0) of 2.0. This value was chosen as it was the highest OD registered in this experiment, occurring in some flasks containing growth medium after 2-3 months of growth. The author does not know it if it is possible to increase the cell densities further. It should be noted that there is a logical flaw in these calculations. They assume constant exponential growth at the highest growth rates observed in this study. However at the EC_{50} level of NH_3-N, growth would logically be inhibited by 50 % and thus it is unlikely that you would observe such fast growth rates.

Table 9: Predictions of nutrient uptake. Growth rate (k) shows the fastest growth rate recorded in that flasks over the course of Experiment 8. The area under the curve was calculated by summing the area of trapeziums fitted to the growth curve. The NH_4^+-N decrease column shows the amount of NH_4^+-N removed over the course of Experiment 8. From this it is calculated how much biomass (represented by area on the graph) is required for 1 mg/l NH_4^+-N consumption and this is multiplied by 400 to predict how much biomass/area would be needed to strip 400 mg/l NH_4^+-N. Assuming the fastest growth rate recorded in the flask in this study (column 2) is held constant and an initial optical density of a_0 =2.0, the final column shows the predicted period of time required to strip 400 mg/l NH_4^+-N from a solution.

	Growth rate (k)	**Area under curve**	**NH_4^+-N decrease (mg/l)**	**Area required per 1 mg/l consumption**	**Area required for 400 mg/l consumption**	**t (days)**
GrM-ad BBM (-NP)-AD a	0.568	8.832	41.94	0.211	84.239	5.66
GrM-ad BBM (-NP)-AD b	0.454	7.864	40	0.197	78.647	6.47
GrM-ad BBM (-NP)-AD c	0.424	10.54	60.2	0.174	69.661	6.50

3.15 Nutrient Uptake Prediction

The predicted amount of time for *S. obliquus* to consume 400 mg/l TAN at two different growth rates is shown in Table 10. 0.568 was chosen as a specific growth rate, as it is one of the fastest growth rates recorded in this set of experiments (in Flask GrM-ad BBM(-NP) AD a in Experiment 8) and 1.19 was chosen as it is one of the fastest growth rates recorded in the literature when culturing *S. obliquus* (see Section 4.1.2.2 and Ho et al. (2010)).

Table 10: Amount of time (t) required for *S. obliquus* to consume 400 mg/l TAN at different specific growth rates (k). The calculations used were those shown in Section 3.14 and Appendix C1, assuming a_0 is 2.000.

	k (d^{-1})	
	0.568	1.19
t (days)	5.66	3.31

These calculations predict that if growth conditions were optimized, the microalgae should be able to removed 400 mg/l TAN within approximately 3 and a half days. This data is discussed in Section 4.3.4. It should be noted that this is an idealized prediction that does not take factors such as self-shading into account.

4. Discussion

4.1 Assessment of microalgae growth on anaerobic digestate liquor

Scenedesmus obliquus was able to grow on low concentrations (1-5 %) of unbuffered ADL, however the growth rate was reduced in comparison to growth medium (see Figure 10, Figure 14 and Figure 15). At a concentration of 10 %, a long lag time of 8-15 days was present before growth occurred, again with a reduced growth rate compared to *S. obliquus* growing in growth medium (Figure 10, Figure 14, Figure 15 and Figure 25). No growth was recorded in concentrations of ADL greater than 10 % (see Figure 10, Figure 12 and Figure 39). The microalgae was found to be unable to grow in high concentrations of digestate (> 10 %), and this follows the general pattern reported in the literature of low concentrations of ADL being used as growth substrates for microalgae (Bjornsson et al., 2013, Cai et al., 2013b, Cho et al., 2013). However, the length of the lag time in the 10 % dilutions was unusual (see Section 4.4). It is typically found that high ammonium concentrations make it difficult for microalgae to grow in raw ADL, while dilutions between 1-10 % ADL make ideal substrates (Bjornsson et al., 2013, Cai et al., 2013b, Cho et al., 2013). This is due to ammonia toxicity (Cho et al., 2013) and insufficient light penetration preventing photosynthesis (Sahu et al., 2013, Kenny and Flynn, 2015). Bjornsson et al. (2013) grew *Scenedesmus* sp AMDD on of different forms of ADL (supplied from vegetable wastes, cow manure, swine manure and *Nannochloropsis granulata*). The ADL was diluted down to ensure the ammonia concentration was 1.5×10^{-3} mol/l NH_3-N (approximately 23 mg/l NH_3-N and 550 mg/l TN), which corresponded to 1 – 6.5 % depending on the ADL. Cai et al. (2013b) grew *Nannochloropsis salina* on ADL with municipal wastewater as a feedstock and recorded maximum growth rates at 6 % loading (160 mg/l TN) and decreased growth at 24 % loading (640 mg/l TN). Cho et al. (2013) attempted to grow *Chlorella* sp. ADE5 on ADL from a WWTP at several dilution ratios (2-20 times). Growth was inhibited in raw ADL and found a 10 % loading (250 mg/l TN) was suitable.

4.1.1 Success at aseptic culturing

As the author was new to aseptic culturing there was the possibility that incorrect technique had led to contamination. The growth curves measured in BBM in Experiment 1 (Figure 10) are characteristic of a single uncontaminated microalgae species, supporting the fact that the microalgae had been cultured aseptically without contaminating the flasks (John Bothwell, Durham University, personal communication, 2014). Additionally, Experiment 5 found no evidence of contamination (Only microalgae grew on the BBM plates. Additionally no bacterial colonies were observed on the streak and spread plates (made with BBM agar) on which *S. obliquus* was cultured, which were kept in the growth room at 25 °C.

Table 7). However, microscopy undertaken in Experiment 7 (Section 3.8) and TEM analysis in Experiment 8 (Figure 33a to Figure 37b) found evidence of contamination. It is possible to see from TEM images that there is little to no contamination in flasks containing BBM or ADL with supplemented trace elements. However there is a lot of bacterial contamination present in flasks containing just ADL and water. This suggests that there are in fact low levels of bacterial contamination in all of the cultures and the degree of proliferation is determined by the medium in the flask. It can be hypothesized that microalgae cannot grow properly in ADL without trace element supplementation, resulting in high numbers of dead microalgae cells which the bacteria can feed on. Alternatively the ADL used in this study (without trace element supplementation) may be a better environment for the bacteria to grow in. Further work would be required to determine why there is heavy bacterial contamination in some cultures and not others. Additionally it is not possible to determine from the current data how and if the bacteria affected microalgae growth. It is possible to see microalgae in flasks with slower growth rates and more dead cells contained more bacteria. However it is not possible to determine whether the bacteria caused cell death/inhibition, or were a product of the availability of dead microalgae cells, or were able to flourish due to reduced nutrient competition from the microalgae or whether it was simply a coincidence. Bacteria can attack microalgae, however certain studies show that symbiotic relationships between microalgae and bacteria in organic substrates can be beneficial for growth (de-Bashan et al., 2004, Cho et al., 2013). Kim et al. (2011) found that certain bacteria can induce flocculation in *Scenedesmus* species – a possible reason for the flocculation seen in heavily contaminated 10%- adapted cultures in Experiment 8 (Figure 38d). If microalgae bioremediation were to be implemented at an NWL STW it would not be possible to keep the cultures sterile, however it is worth noting that contamination may have affected growth rates, compared to other aseptic studies in the literature.

4.1.2 Suitability of growth conditions

4.1.2.1 Growth curve shape

The microalgae show typically shaped growth curves when growing in growth medium. This demonstrates that the conditions in the growth room are satisfactory and are not dramatically inhibitory.

4.1.2.2 Growth rate

Table 8 shows growth rates of microalgae growing in growth medium in Experiments 1, 4 and 8 (between the beginning of the experiment and specific days in the experiment to get a more representative idea of growth rate over the experiment). The highest recorded growth rates in each experiment were 0.381 (d^{-1}) for Experiment 1, 0.356 (d^{-1}) in Experiment 4 and 0.445 (d^{-1}) in Experiment 8 (see Appendix C for calculations). These values are higher than some growth rates that have been reported in the literature (although these were typically measured on a daily basis rather than over an entire experiment). de Morais and Costa (2007) reported maximum specific growth rates of *S. obliquus* between 0.07-0.15 (d^{-1}) in different sections of flat paneled photobioreactors. Xu et al. (2012) also reported low specific growth rate values in *S. obliquus*, ranging from 0.218-0.243 (d^{-1}) based on temperature (between 14 °C and 30 °C). These were lower than the growth rates show Table 8 (0.381-0.445 d^{-1}), however, much higher specific growth rates have been achieved in the

literature. Çelekli et al. (2008) found the specific growth rate of *Scenedesmus obliquus* varied between 0.30-1.02 (d^{-1}) depending on PO_4 and NO_3 concentrations. Ho et al. (2010) obtained a maximum specific growth rate of 1.19 (d^{-1}) in *S. obliquus* when bubbling cultures with 10 % CO_2. In this study dissolved inorganic carbon levels were not measured explicitly, however the lack of CO_2 bubbling likely adversely affected growth rates. Additionally microalgae growth may have become inhibited through the growth experiments due to light limitation caused by self-shading (Kenny and Flynn, 2015). To optimize nutrient uptake, it would be necessary to identify whether certain ambient conditions such as light intensity, illumination structure and carbon source could be optimized to increase the microalgae growth rate.

4.1.2.3 Carbon source

No experiments were carried out during this study to determine source of carbon in the ADL. If the experiments were repeated it would be desirable to measure dissolved inorganic carbon concentration. AD typically has a low C:N ratio (Moeller and Mueller, 2012) and contains high levels of organic carbon. Autotrophic microalgae require an inorganic carbon source to photosynthesize and between pH 7-9 this is mostly present as CO_2 and HCO_3^- (as pH increases more CO_2 is converted to HCO_3^-) (Becker, 1994). *Scenedesmus obliquus* has two dissolved inorganic carbon (DIC)-concentrating systems (for CO_2 and HCO_3^-) as well as CO_2 diffusion, so has the ability to utilize both forms of inorganic carbon at a range of pH (Thielmann et al., 1990). CO_2 dissolving into an alkali solution can act to neutralize it when carbonate/bicarbonate is formed.

However *Scenedesmus obliquus* can also grow mixotrophically and thus can utilize both organic and inorganic carbon sources (Becker, 1994, Abeliovich and Weisman, 1978). Typically this means that the microalgae consume CO_2 in the light and feed on organic carbon in the dark. However as constant illumination was present during this set of experiments, it is unclear whether the microalgae would have been able to consume mixotrophically in the presence of light. It would likely be counter-productive to change the supply of organic carbon to microalgae growing on ADL to try and increase the growth rate (Devgoswami et al., 2011). However supplementing microalgae cultures with CO_2 can increase growth rate (Becker, 1994, Azov, 1982) and previous studies have found that *Scenedesmus obliquus* shows increased biomass productivity when supplemented with 10-15 % CO_2 (Ho et al., 2010, Kaewkannetra et al., 2012). Ho et al. (2010) achieved maximum biomass concentration, biomass productivity and specific growth rate when aerating a 1 l culture vessel with 10 % CO_2 at a flow rate of 0.003 vvm (compared to a range of concentrations between 5-70 % CO_2). Kaewkannetra et al. (2012) achieved a maximum cumulative biomass when aerating 2 l cultures of *S. obliquus* with 15 % CO_2 at a rate of 600 ml/min (compared to aeration with 5 % and 10 % CO_2). As excess CO_2 is available at Bran Sands STW from the present anaerobic digester, this would be an ideal way to increase growth rate.

4.1.2.4 Aggregation

In a number of flasks throughout the experiment in this study, the ADL formed aggregates that interfered with OD measurements. This was especially evident in Experiments 1, 3, 8 and 9. There are a number of potential reasons for the formation of these aggregates. Firstly the aggregates tended

to form in higher concentrations of ADL. So in all likelihood the main factor was that higher ADL solutions contained more particles, so had a higher probability of aggregating together. Secondly, the aggregation may have been linked to the presence of bacteria. In Experiment 8 the heavily contaminated 10%-ad cultures formed large aggregates, whilst the less contaminated GrM-ad cultures did not. However it should be noted that there was no evidence of bacterial contamination in cultures before Experiment 6 (Section 3.6 and 3.8), so this does not necessarily explain the aggregation seen in Experiments 1 and 3. Finally, there is also a possibility that a substance secreted by the microalgae encouraged the aggregates to form. In Experiment 1, a number of aggregates occurred in flasks containing microalgae and ADL concentrations > 40 %, whilst no aggregates were present in the neat ADL containing no microalgae. Overall aggregates would be unlikely to cause an issue for NWL if they implemented a large scale bioremediation system, as the presence of aggregates did not appear to have a dramatic effect on nutrient uptake (Figure 31 and Figure 32) and it would not be necessary to measure the OD accurately.

4.2 Causes of inhibition in Scenedesmus *obliquus* grown on anaerobic digestate liquor

The large differences in growth rate between cultures grown in BBM and various concentrations of ADL suggest that the ADL itself is preventing growth. Possible reasons for reduced growth are:

1) Light
2) pH inhibition
3) Toxic compounds
4) Missing nutrients

There is an obvious trend in the results, showing that increased concentrations of ADL lead to reduced growth. No growth has been recorded in solutions containing > 10 % ADL. This strongly suggests that there is something in the ADL inhibiting growth. When observing microalgae previously grown in BBM, sub-cultured into various dilutions of ADL (Figure 15 in Experiment 4), it would appear that 1 % is the easiest solution for it to grow in. It resulted in the highest growth rate of all the dilutions (0.591 d^{-1} on day 2, Figure 61) with no discernible lag phase. The growth rate is not as high as GrM-ad GrM (0.860 d^{-1}) and the growth rate levelled off much more quickly in the ADL dilutions than in BBM flasks (Figure 61, Appendix D), nevertheless high growth rates were still achieved. This suggests that 1 % ADL does not contain inhibitory levels of a toxin/growth inhibitor and contains enough micronutrients to allow growth (albeit at a reduced rate).

Light is crucial for microalgae growth, however light inhibition is unlikely to be cause of the long lag phases and reduced growth rates observed in ADL dilutions in this set of experiments. Light inhibition could not have caused a long lag phase, as the shading caused by the ADL did not change over the course of the experiments (Figure 10). Additionally in Experiment 8, the lag typically observed in 10 % ADL cultures disappeared once the pH had been neutralized, and the decreased growth rates observed in 10 % ADL cultures also vanished once extra nutrients were supplemented (Figure 28). As this growth occurred in 10 % ADL dilutions, it demonstrates that light inhibition was

not the cause of the long lag phases or the reduced growth rates typically observed in flasks containing ADL. However it may have been a factor in slowing exponential growth once it had begun due to self-shading (Kenny and Flynn, 2015).

4.2.1 Discussing whether the anaerobic digestate liquor toxic or missing trace elements

Experiment 8 was designed to test which of the three factors (pH, toxicity or missing nutrients) could be causing the inhibition.

1. If the microalgae were being inhibited by the pH, or a toxin controlled by pH, lowering the pH alone to 7 would enhance growth to levels equal to that seen in growth medium
2. If a non-pH dependent toxin was inhibiting the microalgae, no increase in growth should be seen from lowering the pH or supplementing the solutions with trace elements.
3. If low levels of micronutrients were inhibiting the microalgae, supplementing the solutions with trace elements should increase growth (although this would not explain growth inhibition increasing with concentration of ADL).

Figure 28 shows 10 % ADL diluted with DI water that was buffered at pH to 7 and it can be seen that there is no lag. The fact that lowering the pH to 7 eliminated the lag strongly supports the theory that the high pH, or a pH-dependent toxin, was causing the lag and growth inhibition.

4.2.2 Determining whether the pH levels are inhibitory

It is unlikely that the range of pH observed in this study (in Experiments 3, 4, 6 and 9) is intrinsically inhibitory to *S. obliquus*. This study has recorded rapid growth occurring in growth medium between pH 6 and 9.25 (Figure 16). Additionally *Scenedesmus obliquus* are documented as being able to survive a wide range of pH (see Section 2.4). Azov and Goldman (1982) stated that pH had no direct negative effect on the growth of *Scenedesmus obliquus*. However, the pH did determine the magnitude of inhibitory growth caused by the presence of free ammonia.

Additionally, as pH changes, bioavailability of trace metals and other compounds may change (Bohutskyi et al., 2014). The pH also affects inorganic carbon speciation (Azov, 1982). At pH > 9 inorganic carbon is mostly available in the form of bicarbonate, while at pH 7 most inorganic carbon is available in the form of CO_2. It was found that the rate of carbon uptake is greater when *Scenedesmus obliquus* was adapted to a low CO_2 environment and then placed into an environment with high CO_2 (i.e. a low pH) (Azov, 1982). *Scenedesmus obliquus* can continue to photosynthesis over a pH of 8, due to a DIC pump for HCO_3^- (Thielmann et al., 1990). However it is not ideal and greatest growth occurs at high CO_2 levels (Azov, 1982).

4.2.3 The effect of ammonium/ammonia speciation and levels

Further tests would be required to determine the exact magnitude of inhibition caused by each factor influenced by pH (bioavailability of trace elements, ammonia/ammonium concentrations or carbon source). However, it is very likely that the high levels of ammonium in the ADL are causing significant growth inhibition. Figure 7, Figure 8 and Figure 9 show the nutrient variation between different batches of ADL and BBM. The greatest concentration difference between the ADL and BBM is in the ammonium concentrations (close to 0 mg/l NH_4^+ in BBM and greater than 1000 mg/l NH_4^+ in the

ADL). This ADL is typical in its high ammonium concentrations (Moeller and Mueller, 2012) and other studies have struggled with microalgae being affected by ammonia toxicity when growing in high concentrations of ADL (see Collos and Harrison (2014) and references within).

As pH and temperature increase, greater concentrations of ammonium ions (NH_4^+) change speciation to ammonia ions (NH_3) (see Figure 44 and FDEP (2001)). The high pH of the ADL (8-9) means that large concentrations of free ammonia will be present in the ADL. This is a problem as free ammonia is more toxic than ammonium. Ammonia is uncharged and lipid soluble, so it can easily diffuse across membranes, while charged ammonium cannot do this (Kleiner, 1981). It is well documented that ammonia causes toxicity in algae (Collos and Harrison, 2014). Abeliovich and Azov (1976) found that at concentrations greater than 2.0 mM (36 mg/l NH_4^+) and pH values greater than 8.0, the photosynthesis and growth of *Scenedesmus obliquus* was inhibited. *Chlorella pyrenoidosa, Anacystis nidulans* and *Plectonema boryanum* were also susceptible to ammonia inhibition. In comparison, the total ammonia nitrogen (TAN) (NH_3-N + NH_4-N) concentration of ADL collected from Bran Sands was over 1000 mg/l (Figure 7, Figure 8 and Figure 9) and in 10 % dilutions the TAN concentration was approximately 110 mg/l (Figure 43c). The ammonia concentration in a flask containing 10 % ADL varies from 5 mg/l NH_3-N at pH 7 to 32 mg/l NH_3-N at pH 9 (Figure 43c).

Azov and Goldman (1982) investigated the effect of free NH_3 inhibition on short-term photosynthesis in three microalgae species; *Scenedesmus obliquus, Phaeodactylum tricornutum* and *Dunaliella tertiolecta.* They performed a series of assays at various concentrations of NH_4Cl and pH. The results showed that the inhibitory compound was free NH_3 and that the only role that pH played in determining the magnitude of inhibition was establishing the degree of dissociation of nontoxic NH_4^+ to toxic NH_3. Azov and Goldman (1982) found that once corrections had been made for pH, all three species displayed the same sigmoidal response curve to free NH_3 concentration; 1.2 mM (16.8 mg/l) of NH_3 resulted in a 50 % reduction in photoassimilation of ^{14}C. The authors suggested that the impact of this finding would be most relevant to low alkalinity freshwaters and intensive algal cultures in which NH_4^+ is the main source of N.

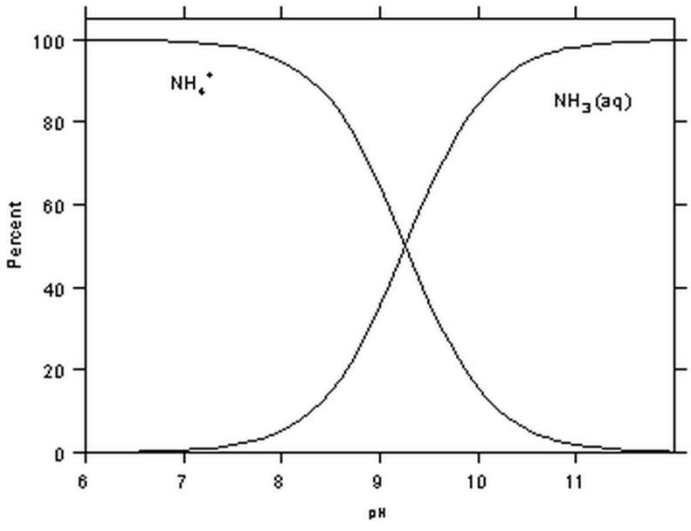

Figure 44: Speciation of ammonia (NH_3) and ammonium (NH_4^+) ions as a function of pH. Source: Illinois State Water Survey (no date)

4.2.3.1 Mechanism of microalgae cell damage by free ammonia

It is well documented that high concentrations of ammonium/ammonia are toxic, but the mechanism through the cell is damaged is still under discussion (Collos and Harrison, 2014, Britto and

Kronzucker, 2002). Ammonium is taken up from the surrounding environment by Amt (a type protein transporter). Amt activity is regulated by the internal nitrogen status of the cell, so is inhibited in ammonium-rich conditions to avoid excess accumulation of intracellular ammonium (von Wiren and Merrick, 2004). However, as ammonia is uncharged, it can freely diffuse through the membrane and the cells are not able to control its uptake (Kleiner, 1981). Cell lysis can occur at extremely high concentrations of ammonium/ammonia (Nagasoe et al., 2010). Until recently it was thought that ammonia toxicity was due to the uncoupling of photophosphorylation (Britto and Kronzucker, 2002). However recent research suggests that ammonia causes damage to the photosystem (PS) II (Drath et al., 2008, Dai et al., 2014).

A key protein within the PSII is the D1 protein, which is sensitive to light-induced damaged and thus a D1 repair process is in place within photosynthetic cells. The studies by Drath et al. (2008) and Dai et al. (2014)showed that ammonia accelerates destruction of the D1 protein, meaning that the presence of ammonia accelerated PSII photodamage even at low light intensities. Ammonium had no detrimental effect on the recovery of PSII from photodamage it simply accelerated the photodamage. To compensate for the increased photodamage, the cell has to elevate expression of ftsH2 (a protease involved in D1 replacement) to carry out an efficient PSII repair cycle (Drath et al., 2008, Dai et al., 2014). However, excess concentrations of ammonia with high photon flux densities (the study used 20 μmol photos m^{-2} s^{-1}) make it near impossible for the cell to repair its PSII system fast enough. Additionally there is a positive feedback cycle, in which a culture of paler cells means less shading and thus each cell is exposed to more light. This means that choosing to constantly illuminate the cultures in this study probably had a detrimental effect and may partially explain the bleached colour of microalgae growing in ADL in Figure 38a and Figure 38c (colour is discussed further in Section 4.2.4).

4.2.3.2 Threshold ammonia/ammonium levels

The toxicity of ammonia to different species of microalgae is shown in Table 11. The EC_{50} of *S. obliquus* is calculated to be 1258 μM, or 17.63 mg/l NH_3-N. Bjornsson et al. (2013) chose 1500 μM NH_3-N, or 23 mg/l NH_3-N, as an appropriate level of free ammonia for *Scenedesmus* sp. AMDD, based on previous work done by McGinn et al. (2012).

Table 11: EC_{50} of different species of Chlorophyceae growing at varying pHs in the literature (sourced from Collos and Harrison (2014)). Estimates of EC_{50} values (for growth, except where noted) for $NH_4 + NH_3$ (total ammonia) and for NH_3 only calculated from final pH values for cultures growing at various PAR values, temperature and at a range of total ammonia concentrations. In some studies growth on NO_3 was the control. Chla: *Chlamydomonas*; Chlo: *Chlorella*; D: *Dunaliella*; N: *Nephroselmis*; NA: not available; NS: not significant; Sce: *Scenedesmus*; Stim: stimulation of growth my ammonium; PAR: photosynthetically available radiation in µmol photons $m^{-2}s^{-1}$.

Genus/species	PAR	Temp. (°C)	pH		$NH_4 + NH_3$ (µM)	NH_3 (µM)	*n*	EC_{50}	EC_{50}	Control	References
			Initial	Final				$NH_4 + NH_3$ (µM)	NH_3 (µM)		
Chla. reinhardtii	100	25	NA	NA	100–10,000	NA	3	Stim	Stim		Giordano et al. (2003)
Chlorella sp.	40–100	23–29	7.2	NA	5000–30,000	60–360	3	Stim	Stim		Kim et al. (2012)
Chlo. protothecoides	Dark	28	6.1	4.0	80,000–725000	80–725	6	NS	NS		Shi et al. (2000)
Chlo. vulgaris	250	18–22	5.9	5.7	2400–12,500	1.2–6.3	5	8046	3	NO_3	Pratt and Fong (1940)
Chlo. vulgaris	250	18–22	5.2	3.7	2400–12,500	1.2–6.3	5	3805	3	NO_3	Pratt and Fong (1940)
Chlo. vulgaris	250	18–22	4.5	3.0	2400–12,500	1.2–6.3	5	6333	3	NO_3	Pratt and Fong (1940)
Chlo. vulgaris	250	18–22	3.8	3.2	2400–12,500	1.2–6.3	5	5622	3	NO_3	Pratt and Fong (1940)
Chlo. vulgaris A23	100	28 ± 2	7.0	4.0	10,000–71,428	70–500	8	31,622	210		Przytocka-Jusiak et al. (1977)
Chlo. vulgaris AA	100	28 ± 2	7.0	8.0	10,000–143000	70–1250	8	56,234	562		Przytocka-Jusiak et al. (1977)
Chlo. vulgaris	70	20 ± 2	7.0	7.0	714–71,428	3–286	10	NS	NS	NO_3	Tam and Wong (1996)
Dunaliella sp.	200–340	20	NA	NA	25–200	NA	8	NS	NS	NO_3	Thomas et al. (1980)
D. salina	60	30	7.6	NA	10–5000	0.3–1.5	5	Stim	Stim		Norici et al. (2002)
D. tertiolecta CCAP	65	18	7.0	7.0	250–16,000	1–48	7	NS	NS		Fabregas et al. (1989)
D. tertiolecta UTEX	NA	23	7.8	NA	1000–50,000	40–2000	4	4536	225	NO_3	Chen et al. (2011)
N. pyriformis	35	20 ± 1	8.7	8.7	1–40	0.2–8	7	224	2	NO_3	Källqvist and Svenson (2003)
Sce. acuminatus	200	NA	7.5	NA	7000–70,000	NA	6	NA	NA		Park et al. (2010)
Sce. obliquus	10	30	9.8	9.2	900–4000	730–3240	6	3162	1258	NO_3	Abeliovich and Azov (1976)

The results in Figure 43a show that even at pH 9 the 1 % ADL dilution is well below the NH_3-N EC_{50} level, so it would not have been affected by ammonia toxicity. This may explain why there was no lag at 1 % dilutions (discussed further in Section 4.4). It also suggests that the difference in growth rates between 1 % ADL and BBM is not due to ammonia toxicity and is likely due to lack of trace elements (discussed further in Section 4.2.4) or another unknown factor. The results in Figure 43c suggest that it would be possible to grow microalgae on 10 % ADL dilution if the pH were controlled, however it is unlikely that microalgae would be able to grow rapidly in neat ADL (Figure 43d).

Trials carried out in the UK on food waste ADL found that *Scenedesmus obliquus* grows rapidly in wastewater/AD diluted to 50 mg/l NH_4-N, although higher concentrations can be remediated if the digestate has been effectively centrifuged and thus there is minimal light inhibition (Phillippe Mozzanega, University of Bath, personal communication, 2013). Cai et al. (2013b) found that *Nannochloropsis salina* grew well on ADL diluted with DI water to 3 %, 6 %, 12 % and 18 % effluent loadings (when undiluted sludge contained 2667±30 mg/l TN). This corresponds to concentrations of 80, 160, 320, 480 and 640 mg/l TN. The highest biomass productivity was observed at 6 % (approximately 160 mg/l TN). Decreased growth was observed at 24 % loading (approximately 640 mg/l TN) and a lag of 4 days was observed (the pH was uncontrolled). Cho et al. (2013) found that optimal growth of *Chlorella* sp ADE5 was obtained on 10 % municipal ADL mixed with 90 % "CR" (a conflux of wastewaters rejected from sludge concentrate tanks and dewatering facilities). This contained 250 mg/l TN and the pH was kept constant at pH 7. Bjornsson et al. (2013) measured growth on diluted swine manure ADL, diluting the effluent with distilled water, distilled water with added magnesium and lake water. The ADL was consistently diluted to 1.5×10^{-3} mol/l NH_3-N (approximately 23 mg/l NH_3-N and 550 mg/l TN). It was found that optimal growth levels were achieved at these dilutions as long as sufficient Mg^{2+} was present.

4.2.4 The effect of missing trace elements on microalgae growth

Figure 28 shows that microalgae growing in 10 % ADL and water that was pH buffered had a slower growth rate than microalgae in BBM. However, the solutions containing 10 % ADL and 90 % growth medium had similar growth rates to pure growth medium. This proves that once the pH has been reduced (thus reducing the concentration of free ammonia), the ADL did not contain anything inhibitory; it is simply missing certain nutrient that were provided in the growth medium. This was further confirmed by the fact that microalgae growing in solutions containing 10 % ADL and modified growth medium (missing nitrate and phosphate see Section 2.5) had similar growth rates to pure growth medium.

Normalized growth rates from 10 % cultures in 3 experiments are compared in Figure 42. "Experiment 4 GrM-ad 10%" had the slowest growth rate (max = 0.111) and did not have its pH buffered or any trace element supplementation. "Experiment 8 GrM-ad H_2O-AD" had an initially high growth rate (0.512 d^{-1}) however due to a decrease in growth rates after day 5, it also had the second lowest biomass productivity of the four cultures (by the end of 14 days). It did not have trace element supplementation, but the pH was kept low with a buffer. "Experiment 8 GrM-ad BBM-(-NP)-AD" also had a maximum growth rate of 0.512 d^{-1}, however the growth rates did not decrease as drastically after day 5 and by the end of 14 days, the OD is three times higher than that of "Experiment 8 GrM-ad H_2O-AD". "Experiment 8 GrM-ad BBM-(-NP)-AD" had been supplemented with trace elements and the pH was buffered to ensure it was below 8. Comparison between "Experiment 8 GrM-ad H_2O-AD" and "Experiment 8 GrM-ad BBM (-NP)-AD" shows that additions of trace elements can triple the productivity of the microalgae. "Experiment 1 10 %" was unbuffered and did not intentionally have trace element supplementation. Its maximum growth rate was 0.264 d^{-1}; higher than Experiment 4 but lower than Experiment 8. Yet its biomass accumulation was similar to a culture that had been supplemented with trace elements. The slow growth rate of 0.264 d^{-1} was likely due to the presence of ammonia. The fact that it was possible for the culture to achieve such a high biomass was likely due to the fact that 3 ml of growth medium containing microalgae was added at the beginning of the experiment, and this may have provided the necessary trace elements to allow this rapid growth to occur.

The TEM results further support the conclusions that both a low pH and trace element supplementation are required for the microalgae to grow well in the ADL. Figure 34a and Figure 34b show one of the parent microalgae cultures (grown in BBM) before it was sub-cultured into the BBM-adapted cultures (GrM-ad) used in Experiment 8. As it was an old culture, it is likely that the circles are lipid stores that have accumulated due to nitrogen depletion in the medium. It is also possible to see that it is morphologically very different from the parent 10 % culture (used in the 10%-adapted cultures in Experiment 8) and that there is much more cell debris and bacterial contamination in the 10 % culture in Figure 33a, Figure 33b and Figure 33c.

GrM-ad microalgae sub-cultured into growth medium are shown in Figure 37a and Figure 37b. Most of the cells are oval shaped and contain one very large chloroplast. No bacteria can be seen in the

sample. The microalgae in Figure 36a and Figure 36b look nearly identical to the cells in Figure 37a and Figure 37b. Most cells in the "BBM-(-NP)-AD" flasks were oval shaped and contained one large chloroplast. There was also no visible contamination. In contrast the microalgae in the "GrM-ad H_2O-AD" flask (Figure 35a and Figure 35b) was mostly round, there was cell debris present, the chloroplast is thin and takes up a small volume of the cell and there is a large amount of bacterial contamination. The absence of certain trace elements from the ADL leads to morphological changes, limited growth and proliferation of bacteria (presumably due to cell death).

Figure 38a, Figure 38b and Figure 38c show that colours of the cultures in four flasks grown in Experiment 8 are different. It would have been beneficial to carry out chlorophyll analysis to better understand the biological reasons behind the colour changes, however it was not possible to carry out this kind of analysis (discussed in Appendix Aii). The flask containing 10 % ADL and DI water was a yellowy green, while the other flasks (BBM-AD, BBM (-NP) AD and H_2O-BBM) were dark green. This was the case through all the experiments; flasks containing dilutions of ADL were yellowy green whilst flasks containing growth medium were dark green. As this was the case through the entirety of multiple experiments, the colour change must have been due to cell colour, not cell density. Figure 38c shows the settled cells to further demonstrate that the colour change is due to cell colour, not the colour of the solution. The fact that the flask containing ADL diluted with trace elements (BBM (-NP) AD) was dark green and the flask containing 10 % ADL diluted with DI water was yellowy green suggests that the colour change is likely due to trace element deficiency rather than ammonia toxicity (unless certain trace elements are required to recover from ammonia toxicity).

4.2.5 Identifying limiting trace elements in anaerobic digestate liquor

It is difficult to determine exactly which trace elements inhibit growth, as only a limited number of ions have been measured. However it is likely that magnesium deficiency was an issue in many of the ADL dilutions. It is typical for magnesium and calcium concentrations to be low in ADL, as the high pH causes them to precipitate out as Ca- and Mg-phosphates (Moeller and Mueller, 2012). Becker (1994) states that in many chlorophyceae, magnesium deficiency can interrupt cell division leading to abnormally large etoliated cells. This is similar to the state of the cells in the 10 % ADL when viewed under the microscope in Experiment 5. Bjornsson et al. (2013) found that microalgae would not grow in swine manure anaerobic digestate without magnesium supplementation with $MgSO_4$ and $MgCl_2$ to 3.04×10^{-4} mol/l (7.4 mg/l Mg^{2+}), similar to the amount found in growth medium (7.51 mg/l - Figure 7). Figure 30 shows that only 0.05-0.2 mg/l Mg^{2+} was recorded in 10 % ADL samples diluted with DI water and the mean concentration of magnesium in neat ADL was of 1.38 mg/l across AD1, AD1703 and AD2901. However magnesium concentrations in European bottled water vary between 1-126 mg/l Mg^{2+} and the mean concentrations in natural waters vary between 8-64 mg/l Mg^{2+} globally (WHO, 2009), so if NWL were to implement a bioremediation system, diluting the ADL with tap water could provide the necessary magnesium levels.

Sulphate is also an essential macronutrient (Grobbelaar, 2004) and although concentrations in neat ADL are similar to those in growth medium (11.39-18.27 mg/l S - Figure 7), concentrations in 1-10 % ADL solutions are much less (approximately 1.90 mg/l S - Figure 31). As the microalgae stripped

approximately 3.50 mg/l S from the growth media over a fortnight (Figure 31) this could have led to inhibited growth in ADL solutions.

It can also be speculated that the ADL does not contain the required levels of iron. It is unknown how much iron is in the ADL as it has not been measured, however bleaching and yellow coloring (as seen in cultures growing in ADL dilutions, see Figure 38a) often indicates iron deficiency (Becker, 1994). To definitively determine which micronutrients were missing from the ADL/causing growth inhibition, further work would need to be carried out. This could include analyzing the ADL to determine which micronutrients are missing and/or setting up trials containing ADL supplemented with certain trace elements and comparing growth to microalgae in growth medium.

4.2.6 Potential toxins within the anaerobic digestate liquor

The ion chromatography results show concentrations of a limited number of ions in the ADL. This makes it difficult to determine whether there could be other toxic compounds in the ADL that could inhibit microalgae growth (at high pH's). Apart from the ammonium, none of the concentrations of the measured ions are in a toxic range. It can be seen from Figure 7 and Figure 8 that there are greater concentrations of sulphate, phosphate, chloride, potassium, nitrite, fluoride, and bromide in growth medium than in the neat ADL. There is no fluoride, nitrite or bromide in the BBM, however it is unlikely that these small concentrations have an inhibitory effect. Only one of the species of microalgae (*Synechococcus leopolienis*) included in a review by Camargo (2003) showed any inhibition at < 50 mg/l F. In fact some species showed growth enhancement at similar concentrations. Therefore the 6 mg/l F present in the ADL is unlikely to have an inhibitory effect. Rao and Sridharan (1980) (cited by Collos and Harrison (2014)) studied the effects of inorganic nitrogen concentrations on benthic estuarine diatoms and found that all three forms of inorganic nitrogen (nitrate, nitrite and ammonium) can become toxic at high concentrations; 17 mM (1054 mg/l) nitrate, 1-10 mM (46-460 mg/l) nitrite and 0.5 mM (9 mg/l) ammonium were inhibitory (mg/l values calculated with respect to whole ion). Thus the 0.12 mg/l of nitrite present in the ADL will not be inhibitory. There is limited published information on bromide toxicity in microalgae. The levels of sulphate, phosphate, chloride and potassium in the neat digestate are 2-3 × higher than in the growth medium. However these ions would not be toxic at a 10 % dilution of ADL.

It is definitely possible that other toxic compounds are present that have not been measured by the ion chromatography technique. For example dodecylethyldimethyl-ammonium bromide is a compound commonly found in wastewater and is toxic to microalgae, with an EC_{50} of 2.63 mg/l in *Scenedesmus intermedius* (Sánchez-Fortún et al., 2008). The bromide in the ADL could indicate the presence of this compound. As the ADL comes from industrial waste, there are a number of other chemicals that could also be present and/or toxic at certain ADL concentrations.

4.3 Nutrient Uptake from anaerobic digestate liquor by *Scenedesmus obliquus*

4.3.1 Comparison with N and P uptake in the literature

In Experiment 8 most of the cultures had approximately 50 mg/l TN and 7 mg/l PO_4-P stripped from them (Figure 31, Figure 32 and Appendix E). It can be seen that microalgae preferentially consume ammonium over nitrate when both are available (see Figure 31 and Figure 32) as suggested in the literature (Maestrini et al., 1986, Collos and Harrison, 2014). In the unbuffered cultures in Experiment 4, approximately 80 mg/l NH_4-N and minimal concentrations of PO_4-P (0-3 mg/l PO_4-P) were removed from the 10 % ADL dilutions over 35 days (Figure 23 and Figure 24). These values are lower than other examples in the literature. Bjornsson et al. (2013) grew *Scenedesmus* sp. AMDD on swine manure ADL supplemented with magnesium and reported a decrease of approximately 23 mg/l NH_3-N and 20 mg/l PO_4-P over 6 days. As the pH was 7 and the temperature was 22 °C this would correspond to decrease of approximately 545 mg/l TAN (NH_3 speciation is dependent on temperature, pH and TAN concentration – see Section 4.2.3). Uggetti et al. (2014) grew mixed microalgae dominated by *Scenedesmus* species on ADL from a wastewater treatment plant (WWTP) and tap water and reported a decrease of approximately 185 mg/l TN over 7 days. Cho et al., (2013) grew *Chlorella* sp. ADE5 on ADL from a municipal WWTP and wastewaters from sludge-concentrate tanks and dewatering facilities. They reported a decrease of approximately 250 mg/l N and 16 mg/l PO_4-P over 5 days. Cai et al. (2013b) grew *Nannochloropsis salina* on municipal WWTP ADL in semi-continuous batch studies and reported a range of consumption rates; 13.4-56.5 mg/l/day N and 2.3-4.3 mg/l/day P, dependent on the harvesting frequency and harvesting ratio. Light limitation and low optical depths can also decrease microalgae growth and thus nutrient consumption (Kenny and Flynn, 2015).

The higher rates of nutrient remediation present in other studies may be due to a number of factors. Many studies used a greater initial cell density. Cai et al., (2013b) discussed the importance of finding the optimal microalgae harvesting frequency and harvesting ratio to boost growth. Cho et al. (2013) used a species isolated from the wastewater so it was already adapted to the conditions in the ADL. Section 4.1.2.2 discusses the fact that the growth rates achieved in this study were not as high as other studies, which would affect the amount of N and P consumed by the microalgae. Further work could be carried out to identify ways to increase nutrient uptake, to improve the efficiency of a bioremediation system at NWL.

Additionally it should be noted that the relationship between external growth media nutrient concentrations and internal cellular nutrient concentrations is complex. It is difficult subject to predict and understand without knowledge of the internal nutrient concentrations of a cell, thus cellular composition should be measured if further work were carried out based on this study. In the case of phosphate, it is documented that if cells have previously taken up large amounts of P, they would not immediately become P-limited if introduced to a growth media in which external P

concentrations were low. However if internal cellular reserves were low the cultures would have been strongly inhibited by lack of P in the external media (Flynn, 2008).

4.3.2 Ratio of N:P consumption and effect on growth

In Experiment 8, the ratio of N:P consumption in the cultures containing ADL and trace elements is approximately 7 (Appendix E). In these solutions, neither the ammonium nor phosphate had been completely depleted by the end of the experiment, so the microalgae was free to uptake N and P at any ratio. The observed mass consumption ratio of 7:1 was slightly lower than expected, as a previous study found that *S. obliquus* requires a mass N:P ratio of 14:1 to grow without any nutrient limitation (Rhee, 1978), although Suttle and Harrison (1988) found *Scenedesmus* species were able to grow and outcompete other species at mass N:P ratios between 2:1 and 5:1.

This apparent preference of consuming N:P at a ratio of 7:1 is not necessarily ideal as NWL's ADL typically has an N:P of 13:1 (Appendix E). Thus the microalgae are consuming less nitrogen and more phosphorus than is available in the ADL. However the microalgae are flexible and are documented in the literature as being able to grow in a wide variety of N:P ratios, (Rhee, 1978, Arbib et al., 2013a). For example, the mean N:P ratio of the BBM cultures in Experiment 8 was 0.84 (n = 6) and the consumption ratio in those flasks over the course of the growth trial was approximately 5:1 (Appendix E). This low consumption ratio was likely due to luxury uptake of P by the microalgae cells (Mayers et al., 2014) and a response to depletion of nitrogen concentrations. Mayers et al. (2014) investigated the growth of *Nannochloropsis* sp. at different N:P ratios and found that a mass ratio of 14:1 did not compromise productivity and at a lower N:P ratio of 7.2:1, P removal was 1.5-1.7 fold greater but no increase in biomass was observed due to luxury phosphorus uptake. Therefore, it is likely that the consumption ratios observed in this study have been influenced by the high total concentrations of P available and the ADL ratio of 13 would not be detrimental to microalgae growth on a large scale.

4.3.3 Trace element uptake

A great deal of fluctuation was observed in the trace element concentrations over 35 days in Experiment 4 (Figure 17 to Figure 24). It is difficult to determine how much of this fluctuating is due to pH-induced changes (the pH was unbuffered so changed throughout the experiment - Figure 16). Additionally none of the flasks were measured on day 0, instead a sample of neat ADL was measured and the nutrient concentrations were extrapolated from this single nutrient measurement. This means that it is unclear whether nutrient contamination was present from previous cultures (see Section 4.4.4.3). Therefore the trends seen in the nutrient data in Experiment 8 (Figure 30 to Figure 32) are more reliable, as the pH was buffered and measurements were taken on day 0 and at the end of the experiment (day 14).

The most important trend to highlight is the change in magnesium concentrations in Experiment 8. Approximately 2 mg/l of magnesium was consumed in each of the cultures that contained trace elements (Figure 30 and Appendix E2). However in the cultures containing only 10 % ADL and DI water, little over 0.1 mg/l Mg^{2+} is present at the beginning of the experiment and by the end the

concentrations in the solutions had increased to approximately 1.4 mg/l Mg^{2+}. Magnesium is an essential trace element (Becker, 1994) and it is feasible that without sufficient concentrations of magnesium and other crucial trace elements, many microalgae cells died and thus release their micronutrients. Cell death could also explain the excess cell debris and high number of bacteria seen in the TEM images of microalgae growing in solutions of ADL and DI water (Figure 35a).

From Figure 30, Figure 31 and Figure 32, it can be seen that fluoride and calcium concentrations typically increased in all cultures; bromide and sulphate concentrations decreased; and chloride, sodium and potassium concentration fluctuations were not significant when standard errors were taken into account (Appendix E2). Sulphate concentrations likely decreased due to their status as an essential micronutrient (Becker, 1994) and it is logical that the sodium and chloride concentrations would not change as they are biologically inert.

4.3.4 Predicted nitrogen uptake

As NWL are interested in whether it is physically and economically feasible to set up a microalgae bioremediation system, it is important to calculate how much of each nutrient could potentially be stripped from the ADL and what resources would be required to do so. Although the aim of this study was to investigate both N and P removal potential by microalgae, the N:P ratios fluctuated a great deal between experiments (Appendix E) so it was only possible to carry out further analysis on one nutrient. It became apparent through the course of the investigation that ammonia concentrations would have to be monitored and controlled to enable maximum growth to occur (see Section 4.2), so the author chose to focus on calculating predicted nitrogen uptake. It appears that free ammonia (NH_3) is the main toxin within the ADL, when it is above a threshold of approximately 17-23 mg/l NH_3-N. At pH 7, this corresponds to a concentration of 400-540 mg/l TAN. Calculations based on the fastest recorded growth and uptake rates recorded in this study predict that the microalgae should be able to strip 400 mg/l TAN from the ADL within 5-6 days (Table 9). Based on this study, the known conditions required for growth rates greater than 0.4 d^{-1} are ammonium concentrations to be lower than 400 mg/l NH_4^+-N, the pH to be buffered to 7 and necessary trace elements to be supplemented.

As the concentration of NH_4^+-N in unautoclaved ADL is approximately 1500 mg/l (Table 15-Appendix A2), the neat ADL would have to be diluted by 3.75 × to achieve a concentration of 400 mg/l. Typically 50-200 m^3 of ADL is produced daily at Bran Sands STW, with peaks of up to 600 m^3/day. Allowing for 200 m^3/day, this would mean that once diluted, NWL would need to have the capacity to store 750 m^3 of diluted liquor for 6 days to allow it all to be remediated. During this time a further 1200 m^3 of neat returns liquor would be generated, so to prevent a backlog, six 750 m^3 photobioreactors/open ponds operating in a batch system would be required to carry out the remediation. Further work would be required to determine whether photobioreactors or open ponds would be more suitable, as growth rates in photobioreactors are usually higher, but open ponds can hold greater volumes of liquid and are cheaper (Leite et al., 2013).

Either increasing the cell density at the beginning of the remediation process, or increasing the growth rate and thus nutrient uptake rate could reduce the amount of time required to remediate

the waste. Changes to the initial OD/cell density do not have a dramatic effect on the time taken for the microalgae to consume 400 mg/l TAN (data not shown) compared to changing the growth rate, which could lead to a substantially reduced remediation time. The growth rates of microalgae in 10 % ADL with pH buffering and trace element supplementation were the same as the growth rates in growth medium (see Section 4.1.2.2). As the growth rates recorded in growth medium in this study (0.381-0.445 d^{-1} – see Table 8) were lower than those recorded in other studies using the same microalgae species, it is reasonable to conclude that the growth conditions may not be optimal and it may be possible to increase the growth rate if the conditions were altered.

Table 10 shows that if the growth rate could be increased to 1.19 d^{-1}, it may be possible to remove 400 mg/l TAN within approximately 3 and a half days. The growth rate of 1.19 d^{-1} was cited by Ho et al. (2010), who were culturing *Scenedesmus obliquus* under various CO_2 concentrations. Therefore, if the microalgae were grown in diluted ADL, with trace element supplementation and pH buffering, but otherwise under the same growth conditions as the microalgae cited in Ho et al. (2010), it may be possible to reduce the remediation time to 3 and a half days, thus only requiring three or four 750 m^3 photobioreactors/open ponds to treat the ADL produced at Bran Sands STW.

However it should be noted that these are idealized calculations that do not take light limitation into account. Microalgae growth can become limited at optical depths greater than 0.1 m, so the area required to remediate 750 m^3 of diluted ADL in open ponds would likely be unfeasible (Kenny and Flynn, 2015). Even within shallow ponds or photobioreactors light limitation may remain an issue. Kenny and Flynn (2015) found from model simulations that microalgae grown on f/2 media (containing 12.35 mg/l N) in a chemostat-style continuous culture were affected by light limitation caused by self-shading. Due to the intrinsic link between biomass production and N uptake, it is unlikely that it would be possible to achieve the consumption of 400 mg/l TAN without limiting growth due to self-shading. Further investigation would be required to determine the maximum N uptake possible without inducing light limitation from self-shading.

4.4 Growth lag times in microalgae cultured on high pH anaerobic digestate liquor

4.4.1 Length of the lag time in comparison to other studies

The lags in initial growth recorded in Experiments 1, 2, 4 and 6 (shown in Figure 10, Figure 11, Figure 15 and Figure 25) were typically around 200 hours, ranging from 8-15 days. The fact that this large lag/inhibition time was reproduced in each experiment shows that it was not an anomaly. Table 12 shows that in the literature there are no reported lag times (between inoculation and exponential growth) of the magnitude of 200 hours. It should be noted that the table shows the lag period between inoculation and the time taken to reach maximum ammonium uptake. Therefore it is not possible to directly compare the lag times in this table, as ammonium uptake was not measured on an hourly or daily basis in this experiment. However it is likely that maximum ammonium uptake occurred during exponential growth, so ammonium uptake may act as an indicator for exponential

growth (which can be compared to onset of exponential growth shown in the OD measurements taken in this experiment).

Table 12: Induction of ammonium uptake by unicellular algae in laboratory cultures on various physiological N states and growing on various N sources (table sourced from Collos and Harrison (2014)). Lag = time to reach V_{max} for ammonium uptake. PAR in µmol photons $m^{-2}s^{-1}$, NA = not available. Nat: natural; Lim: limited; starv: starved; suff: sufficient.

Species	PAR	T (°C)	pH	Previous N source	Physiological status	NH_4 addition (µmol N/l)	Lag (h)	References
Chlamydomonas reinhardii	65	25	7.0	NO_3	N starv. 16 h	900	0	Thacker and Syrett (1972)
Chlamydomonas reinhardii	65	25	7.0	NH_4	N suff.	200	0	Cullimore and Sims (1980)
Chlamydomonas reinhardii	500	25	7.0	NH_4	N suff.	200	0	Florencio and Vega (1983)
Chlorella fusca	70	25	7.4	NH_4NO_3	N starv.16 h	9000	48	Syrett and Morris (1963)
Chlorella fusca	140	25	7.4	NH_4NO_3	N starv. 16 h	9000	24	" " "
Chlorella vulgaris	Dark	25	6.0	NH_4NO_3	N starv. 16 h	10,000	0	Syrett and Fowden (1952)
Ditylum brightwellii	175	15	NA	NO_3	N.A.	1	75	Eppley et al. (1969)
Emiliana huxleyi	150	13	NA	NO_3	N suff.	5	85	Page et al. (1999)
Emiliana huxleyi	150	13	NA	NO_3	N suff.	10	60	" " "
Emiliana huxleyi	150	13	NA	NO_3	N suff.	17	72	" " "
Lingulodinium polyedrum	175	15	NA	NO_3	NA	3	24	Eppley et al. (1969)
Lingulodinium polyedrum	100	18	NA	NO_3	N starv. 24 h	10	0	Harrison (1976)
Haslea ostrearia	100	NA	7.8	NO_3	N suff.	30	48	Robert and Maestrini (1986)
Nitzschia ovalis	100	NA	7.8	NO_3	N suff.	30	48	Robert and Maestrini (1986)
Nitzschia ovalis	nat PAR		NA	NO_3	N starv. 24 h	40	24	Maestrini et al. (1986)
Phaeodactylum tricornutum	NA	NA	7.6–8.0	NO_3	N suff.	200	96	ZoBell (1935)
Phaeodactylum tricornutum	200	20	8.0	NH_4	N suff.	300	0	Cresswell and Syrett (1982)
Platymonas striata	90	20	NA	NH_4	N starv. 1 h	1200	1	Ricketts (1988)
Skeletonema costatum	310	17	NA	NA	N suff.	5	0	Conway (1977)
Skeletonema costatum	490	17	NA	NH_4	N lim.	8	0	Conway (1977)
Skeletonema costatum	NA	13	NA	NO_3	N starv. <24 h	8	24	DeManche et al. (1979)
Thalassiosira antarctica	50	0	NA	NO_3	N suff.	25	0	Döhler (1991)
Thalassiosira antarctica	50	0	NA	NO_3	N suff.	50	2	Döhler (1991)

4.4.2 Possible causes for the long lag time observed in experiments

Three options were hypothesized to explain the long lag time:

1) Changing microalgae (adaption)
2) Changing ADL (pH change, toxin reduction, micronutrient increase)
3) Both (microalgae changing to alter ADL)

4.4.2.1 Microalgae adaption as an explanation for decreased lag times in later experiments

Microalgae are highly adaptive organisms. Lau et al. (1996) acclimated *Chlorella vulgaris* to wastewater for 14 days; increasing chlorophyll content and N and P removal efficiency. Acclimated cells were able to remove 80 % inorganic N and 70 % inorganic P over a 2 day retention time, compared to 54 % inorganic N and 50 % inorganic P removal in unacclimated cells. Stockner and Antia (1976) highlighted the importance of long term bioassays, stating that exposures of 20-40 days can be required for phytoplankton to adapt to a pollutant. They also discussed the fact that phytoplankton can be trained to tolerate high levels of a pollutant by repeated exposure to gradually increasing pollutant concentrations. Przytocka-Jusiak *et al.* (1978) described how they adapted *Chlorella vulgaris* to higher concentrations of ammonium, increasing the EC_{50} of *Chlorella vulgaris* from 330mg/l NH_4-N to 1500mg/l NH_4-N. They achieved this by incubating the microalgae at a high, inhibitory level of ammonium; where no growth occurred but the microalgae did not die, simply remained dormant. The microalgae were then sub-cultured four times into new cultures containing the same inhibitory levels of ammonium. Finally they were sub-cultured back into a solution containing a lower level of ammonium; at which point growth occurred at an ammonium concentration that was previously inhibitory. In this way, the ammonium tolerance of the microalgae

was increased dramatically within 1 month. So adaption is possible, however Przytocka-Jusiak (1977) do not explain how this adaption has occurred in terms of biochemical changes.

It was hypothesized that the growth in Experiment 1 (Figure 10) after an 8-day lag may have been due to adaption allowing the microalgae to grow in inhibitory conditions. When microalgae previously growing on 10 % ADL were subbed into more 10 % ADL in Experiment 2 (Figure 11), the shorter lag time appeared to confirm this adaption hypothesis. However, subsequent tests revealed more complicated results. Experiment 3 (Figure 12) was inconclusive due to aggregate formation (a different ADL with more particulate matter was used) and Experiment 4 (Figure 15) appeared to show that pre-adapted microalgae had a shorter lag time and slower growth rate, while non-adapted microalgae had a longer lag time but faster growth rate (discussed further in Section 4.4.4). Experiment 6 (Figure 25) showed a spread of results, with pre-adapted microalgae doing slightly better, but only just. In Experiment 8 (Figure 28) the lag time was eliminated in pH buffered cultures.

Overall, the experiments did not reliably show that pre-incubating the microalgae in 10 % ADL led to better growth in new solutions of 10 % ADL. These mixed results demonstrated that the issue of growth inhibition in the ADL was too great to overcome by simply exposing successive generations of microalgae to the ADL.

4.4.2.2 Establishing whether the lag be explained by changing anaerobic digestate liquor

4.4.2.2.1 Changes within the anaerobic digestate liquor

For a lag to occur, something has to change: either the microalgae or the environmental conditions. As discussed in Section 4.4.2.1, the results of Experiments 2, 3, 4 and 6 do not appear to support the hypothesis that microalgae adaption caused the lag. Therefore it must (at least partly) be due to changing environmental conditions. The primary inhibition suspect was ammonia toxicity, which is linked to pH, temperature and ammonium concentration. Temperature remained constant throughout the experiments. Nutrient concentration and pH were measured to determine whether they changed over time, and these changes could explain the long inhibited growth phase in 10 % concentrations.

4.4.2.2.2 Nutrient change in un-inoculated anaerobic digestate liquor

IC analysis was repeatedly carried out on three ADL samples over a three-week period to determine whether nutrient concentrations were stable in samples being stored at 4 °C (data in Appendix A2iv). This data shows that over the three weeks, ammonium concentrations remained stable (with a RSD of 5-7 %) in the un-inoculated ADL. This meant that the ammonium concentrations were not decreasing to a non-toxic threshold naturally over a three-week period. It should be noted that there are many other chemicals present in the ADL that it was not possible to test, and this was ADL stored at 4 °C, not the 25 °C used in the growth experiments.

4.4.2.2.3 Nutrient change in inoculated anaerobic digestate liquor

Figure 17 to Figure 24 show that in inoculated growth medium and ADL dilutions, all nutrients changed over time and ammonium concentrations went down in ADL flasks. This demonstrates the

obvious fact that microalgae do change the nutrient environment. No measurements were taken around the 10-day mark when growth usually began to occur in the 10 % flasks, so it is unknown what the conditions were in the flasks at that time.

4.4.3 Effects of changing anaerobic digestate on microalgae growth

4.4.3.1 Observed pH change over time and potential mechanisms

To determine how pH changed over the course of experiments, pH was monitored in Experiments 4 (Figure 16), 6 (Figure 26) and 8 (Figure 29). The general trends observed were that the flasks containing ADL and microalgae started with a high pH (8.00-9.50) and decreased over the experiment to 7.00-8.50 (a decrease of just less than 1 unit was typical). In flasks containing growth medium and microalgae, the pH would initially be low (6.00-7.00) and increase over the experiment (to 8.00-9.00).

4.4.3.2 Establishing whether pH changes were due to chemical or biological factors

The pH of un-inoculated ADL was not measured over a long time period, so it is not possible to quantify what proportion of the pH changes were due to chemical processes and what was due to biological processes. The pH of neat ADL was monitored overnight and little change was seen (data not shown). The pH of ADL dilutions remained relatively similar at the start of each experiment. It would be expected for the pH to drop a certain amount during the experiment due to CO_2 dissolution. However the different trends seen in growth medium and ADL suggest that nitrogen consumption by microalgae are the driving force behind the pH changes. The literature documents that pH increases when nitrate is consumed due to release of OH^- ions and decreases when ammonium is consumed due to release of H^+ ions (Raven and Smith, 1976, Goldman et al., 1982). Figure 29 shows that even in a buffered culture the pH changes depending on the nitrogen source in the flasks – the pH in the nitrate-rich growth medium increased, while the pH in flasks containing ammonium-rich ADL decreased. It should be noted DIC depletion increases the growth media pH (Shiraiwa et al., 1993), which could be an additional factor for the pH increase in the growth medium flasks, however decrease in DIC does not appear to have as much effect on growth medium pH as nitrogen source and uptake. It should also be pointed out that in a commercial system, it would not be financially feasible to use expensive organic buffers, so alternative methods would have to be used to control the pH.

4.4.3.3 Establishing a threshold pH/ammonia level.

A potential contributing reason to the long lag time seen in the 10 % ADL cultures is that once the pH decreased below a certain point, the microalgae grew and the lag period terminated. Figure 27 shows the OD vs pH in Experiment 6. The pH starts high, between pH 9.20-9.30 (at pH 9.20 the calculated NH_3-N concentration is 76 mg/l). No growth occurs above pH 8.90, below this pH growth begins to occur in 5 out of the 6 flasks. At pH 8.87, the NH_3-N concentration is calculated to be 23.02 mg/l. Bjornsson et al. (2013) stated that *S. obliquus* required an NH_3-N concentration less than 23.0 mg/l to be able to grow. Collos and Harrison (2014) state that the EC_{50} ammonia level in *S. obliquus* is 17.62 mg/l NH_3-N – which would have occurred at pH 8.72 in Experiment 6. This data appears to support the theory that the microalgae will remain in the lag phase until the pH has decreased enough to reduce the ammonia concentration below a threshold level.

There is variation in the literature about whether the pH-ammonium relationship has a threshold level that causes a lag. Uggetti et al. (2014) grew mixed species of microalgae, dominated by *Scenedesmus* species, on ADL. The pH was uncontrolled and in fact it was typically between 8 and 9, with high ammonium concentrations of up to 260 mg/l NH_4^+-N. The high concentration of ammonia inhibited the growth rate, but did not cause a lag. However a lag of four days was observed by Cai et al. (2013b) when *Nannochloropsis salina* grew on un-buffered 24 % ADL loadings (approximately 160 mg/l TN) and a one-day lag was observed at lower dilutions.

4.4.4 Possible causes of difference in growth rate and lag time in Experiment 4

The results of Experiment 4 were especially confusing. The experiment aimed to prove, or disprove, the hypothesis that pre-acclimation in ADL improves growth in 10 % ADL. The results shown in Figure 14 and Figure 15 appeared to show that pre-incubation environment does make a difference. However whether that difference was a positive adaption was difficult to determine. The lag time was shorter in the 10%-ad 10% cultures, however the growth rate was much slower, not only in the 10%-ad 10% cultures, in the 1 % and 5 % cultures as well. So if the cultures had adapted, they were definitely not thriving; growth happened, but at a much slower rate. It would appear that the microalgae have been permanently damaged by their time in the ADL. However the 10%-ad microalgae reintroduced to the growth medium grew just as well as the GrM-ad microalgae, so the microalgae obviously had the ability to recover. There are a number of possible reasons for the growth differences observed between the microalgae previously grown in ADL and growth medium, discussed in the sections below.

4.4.4.1 Inter-generational micro-nutrient depletion

Drath et al. (2008) and Dai et al. (2014) found that free ammonia causes rapid photodamage of PSII, so the PSII repair system is required to be more efficient. It is possible that the PSII repair system requires a certain set of trace metals that may not be abundant in the ADL. Bohutskyi et al. (2014) stated that newly generated cells are likely restrained by the reserves present in the parent cells, as well as the fact that intracellular content of many micronutrients decreases rapidly once the nutrient is no longer present in the growth medium. Therefore, if an essential micronutrient was missing from the ADL, the first set of cells put into the ADL may use existing internal reserves to increase expression of ftsH2 and speed up the PSII repair system. As the pH decreases (partially due to dissolution of CO_2 into the solution, though mostly due to consumption of ammonium) and overall ammonium concentrations drop due to N-uptake, free ammonia levels in the ADL would decrease. This would mean that the PSII repair system would not have to be as efficient. However when the cells are sub-cultured a second time into ADL with a higher pH and ammonia levels, the PSII repair system has to speed up again. If the micronutrients needed are not available and intercellular reserves have been depleted, this may be the reason reduced growth is observed in 2nd generation cells compared to 1st generation cells. This would also explain why there is no lag in 2nd generation cells compared to 1st generation cells (the PSII repair system is already functioning at an elevated rate compared to the microalgae cultured in BBM), but the lack of micronutrients prevents it ever

completely recovering, leading to slow, steady growth. It also explains why 10%-ad microalgae makes a full recovery when reintroduced to growth medium; the toxic ammonia is no longer present and the micronutrients needed to repair the PSII system are present. Figure 28 shows that the presence of trace elements made a significant difference to growth rate. Experiment 8 aimed to further investigate whether the presence of trace elements in the pre-incubation environment affected growth rate and lag time. However the lag times in both solutions were reduced to less than 24 hours due to the decrease in pH, so it was not possible to see a difference in lag times on a daily resolution. The extreme flocculation of the 10%-adapted cultures (shown in Figure 38d) also made it impossible to measure and compare the growth rates of 10%-ad and GrM-ad cultures.

4.4.4.2 Contamination

TEM images taken in Experiment 8 show that there is relatively little contamination in cultures containing growth medium (Figure 34a), however once the microalgae was introduced to 10 % ADL cultures the bacteria proliferated (Figure 35a), while remaining at very low levels in flasks containing trace elements (Figure 36a and Figure 37a). While the exact causes of the bacterial proliferation in non-supplemented ADL cultures were not studied within the time frame of this project, here we speculate on possible reasons. There was the possibility that when microalgae (and a minute amount of bacteria) were introduced to a 10 % ADL solution, a significant proportion of the cells died. Other microalgae cells were dormant until it was possible for them to grow (the microalgae adapted and/or the ADL became less inhibitory as the pH decreased). Meanwhile the bacteria fed on the debris of the dead cells. In Experiment 8, the biomass of the parent 10 % ADL culture was composed of 1 % bacterial cells and 99 % microalgae cells. When the 10 % culture was sub-cultured a second time, many microalgae cells died. Numerous bacteria were already present and they multiplied rapidly whilst consuming the new dead cells. However the microalgae could also grow quicker this time, as they were better adapted to the conditions. On the other hand, there were also more bacteria to compete with/be attacked by so this hampered growth – resulting in a shorter lag time and a slower growth rate in 10%-ad cultures compared to GrM-ad cultures. The 10%-ad cultures were not photographed with TEM so it is not possible to visually ascertain whether bacteria made up a greater proportion of the biomass in these cultures. Completely aseptic trials would be required to determine whether contamination made a difference to growth rate, although this knowledge would not benefit NWL as an aseptic treatment system would not be viable due to the unsterile ADL.

4.4.4.3 Nutrient Residue

No nutrient measurements were taken at the beginning of Experiment 4, so there could have been different nutrient and pH levels in GrM-ad and 10%-ad flasks due to the origin of the microalgae (despite rinsing twice). The different pH and nutrient levels could have caused different patterns of growth. However the IC analysis of Experiment 8 showed that rinsing is an efficient technique at removing residual nutrients and there was little difference in nutrient concentrations of growth medium adapted and 10%-adapted cultures (Figure 30 and Figure 31). However as accurate IC and pH measurements were not taken for Experiment 4, this cannot be ruled out as a factor, though seems somewhat unlikely.

4.4.4.4 Cell Density

As algae uptake ammonium as they grow, it stands to reason that a culture containing a large numbers of cells would be at an advantage as toxic ammonia would be stripped out faster. The GrM-ad flasks had a greater cell density than the 10%-ad flasks. This was partly due to the process of rinsing the microalgae. Once a 10 % ADL culture has been centrifuged, it is not possible to tell from OD measurements what proportion of the absorbance is due to microalgae vs. solids. Therefore the target OD in Experiment 4 was 0.05 for GrM-ad cultures and 10%-ad cultures. Miscalculations during inoculation led to the starting OD of 10%-ad flasks being 0.039 and GrM-ad flasks being 0.05. As microalgae grow exponentially, the difference in initial cell numbers can explain a proportion of the difference in growth rates observed between GrM-ad and 10%-ad cultures. This is an issue that could have been avoided if the equipment had been available to carry out continuous culture experiments instead of batch experiments.

4.4.4.5 Unequal Cell Number Removal

There is the possibility that a mistake was made when removing solution for nutrient analysis on day 6 of Experiment 4. There is a distinctive peak in a number of the flasks on day 6 and a reduction the following day. This peak may have been an anomaly, the microalgae may have been stressed or different proportions of the microalgae may have been removed from different solutions accidentally. The flasks were sitting on a bench-top for a while before they were sampled; they were then "swilled" in a circular motion to resuspend any microalgae that may have settled to the bottom of the flask. However, the resuspension may have resulted in concentrating the microalgae in the bottom, centre of the flask, so that a large proportion of the microalgae in the flask were then removed when 10 ml of solution was taken to test nutrient concentrations. If 50 % of the microalgae were removed from one flask and only 20 % removed from another, this could make it appear that the first culture appear to have a slow growth rate, when in fact it was growing at the same rate, but simply had less cells in it. It is not possible to say how much of an impact this may have had, as the proportion of the cells removed is unknown.

4.4.5 Conclusion of cause of growth lag time

The fact that the lag disappeared in Experiment 8 when the pH was buffered to approximately 7, in GrM-ad and 10%-ad flasks, shows that a pH-dependent toxin caused the lag. The literature and nutrients measured in the digestate strongly suggest that the pH-dependent toxin is ammonia. It is possible to speculate that microalgae consumption of ammonium reduces the effect of ammonia toxicity (both by reducing ammonium concentrations and lowering the pH) making the conditions of the ADL more favorable over time. Simultaneously certain physiological changes in the microalgae make them better able to survive and grow in the hostile environment. However the data collected cannot definitively prove this is the case and further work would have to be carried out to do so.

4.5 Unsterile growth in tap water

Experiment 9 aimed to test microalgae growth in more realistic conditions. It planned to investigate whether tap water would provide some of the essential missing trace elements, as it will be cheaper for NWL to use tap water than to supplement the cultures with (potentially expensive) chemicals. The experiment also planned to test whether microalgae could grow in higher ADL concentrations if

the pH was kept at 7 and whether the microalgae growth rates were affected by unsterile conditions. As it was not feasible to set up CO_2 bubbling (owing to restrictions of using bottled gas in the culture room) the pH of the ADL dilutions was lowered with frozen CO_2 (dry ice). The temperature of the ADL during this treatment was not measured. The frozen CO_2 reduced the pH to below 7 and it remained at a low pH overnight. However, once the experiment had begun the pH returned to 9 and no growth occurred (Figure 39 and Figure 41 respectively). The pH was reduced with sodium bicarbonate, however it returned to 9 again the next day. It is possible that the microalgae were stripping the preferential form of carbon out of solution (increasing the pH) more rapidly than the ammonium could be consumed to lower the pH. As it was not possible to maintain the pH below 8, the experiment was ended and it was not possible to answer these questions.

5. Conclusions and Further Work

The work presented within this thesis found that there were two key factors inhibiting the growth of microalgae on ADL: high free ammonia concentrations and low trace element availability. The data suggested that ammonia concentrations greater than 17.5-23.0 mg/l NH_3-N prevented the growth of *Scenedesmus obliquus*, similar to the findings of Collos and Harrison (2014) and Bjornsson et al. (2013). When microalgae were introduced to unbuffered 10 % ADL solutions, the high pH (typically pH 9.30) meant that the NH_4-N:NH_3-N ratio was low (at pH 9.20 concentrations in the ADL were approximately 34 mg/l NH_4^+-N and 76 mg/l NH_3-N (FDEP, 2001)). This meant that the NH_3-N concentration in the ADL was above the inhibition threshold and the cultures couldn't grow. However, when microalgae were sub-cultured into 10 % ADL dilutions buffered to approximately pH 7.00, the concentrations of ammonium and ammonia were 105 mg/l NH_4^+-N and 5 mg/l NH_3-N, and exponential growth began within 24 hours. *S. obliquus* is well known for its ability to grow at a wide range of pH (Goldman et al., 1982, Thielmann et al., 1990) and was observed growing in growth medium at a pH of 9.25 in Experiment 4. Therefore it is almost certain that the lag was caused by pH-induced high ammonia concentrations in unbuffered ADL. In fact when optical density measurements and pH data from Experiment 6 were plotted against calculated ammonia concentrations (Figure 27), it could be seen that pH (and thus ammonia concentrations) steadily decreased over the course of the experiment and exponential growth occurred as the threshold of ammonia toxicity was crossed (approximately 20 mg/l NH_3-N). This steady pH decrease in 10 % ADL flasks during lag periods was recorded in multiple experiments, even though no microalgae growth was occurring. Further work could identify whether this pH decrease was due to dissolution of CO_2 in the ADL; consumption of ammonium by the microalgae whilst in a dormant phase (ammonium consumption causes the release of H^+ ions and thus reduces the pH (Raven and Smith, 1976, Goldman et al., 1982)); or another unknown mechanism. Additional work could also attempt to replicate the long 200 hour lag by supplementing growth medium with high ammonium concentrations, buffering the solution to pH to 9 and inoculating microalgae into the solution.

However the high ammonia concentration was not the only issue affecting microalgae growth, as the productivity of microalgae in 10 % ADL and DI water was approximately 1/3 of that in flasks containing growth medium over a two week period, despite initially high growth rates. Experiment 8 proved that this reduced growth in 10 % ADL flasks was due to missing trace elements, as once a trace element stock solution were added to 10 % ADL, the biomass accumulation was similar to that observed in growth medium (Figure 28). In fact, the ammonium (NH_4^+) was preferentially stripped out of the BBM before the nitrate, illustrating that 10 % ADL with additional trace elements was not just as good as growth medium, it was preferred by the microalgae. Further experiments could be carried out to determine which trace elements were missing from the ADL, and whether they are present in tap water. Once the missing trace elements have been identified, the costs of supplementing them can be analyzed and factored into larger calculations determining whether it was possible for the bioremediation system to be economically viable.

Using 17.62 mg/l NH_3-N as an inhibition threshold for exponential growth, it was calculated that the highest concentration of ADL that *S. obliquus* would be able to grow on is 37 % ADL at pH 7.0 (= 400 mg/l TAN and 17.25 mg/l NH_3-N). Growth trials could be carried out to determine whether this calculation was correct. It was also calculated that it would take *S. obliquus* 5-6 days to strip 400 mg/l TAN from the ADL, based on the nutrient uptake rates and fastest growth rates (0.424 - 0.568 d^{-1}) recorded in this study. However if the growth rate could be increased to 1.19 d^{-1} (reported in Ho et al. (2010)) this remediation time could be reduced to 3.31 days.

Bran Sands STW produces 50-200 m^3 of ADL daily, with peaks of up to 600 m^3/day. It was calculated that to treat 200 m^3/day, NWL would need to have the capacity to store 750 m^3 of diluted ADL for the 3-6 days it was predicted would be required to carry out the remediation process. Additionally three to six 750 m^3 photobioreactors/open ponds would be required to carry out the remediation in batches without creating a backlog. NWL have stated that would be possible to store this volume liquid at the Bran Sands site. However this prediction is likely an over-estimation as it did not consider the impacts of light limitation and further work would be required to address this.

To identify the growth conditions required for growth rates > 1.0 d^{-1}, further work would have to be carried out to determine the ideal growth conditions, e.g. bubble the cultures with various CO_2 concentrations, change the light intensity, light period, pH and temperature conditions. The cost of providing these conditions would also have to be assessed.

There are a number of other issues that would need to be investigated to establish the economic feasibility of using microalgae as a bioremediation system at Bran Sands. The first is the effect of using natural light for microalgae growth. Natural light is the cheapest form of light available and fluctuates throughout the year at temperate latitudes. It has been found that decreased temperatures and light intensity results in lower microalgae growth and uptake of N and P (Boelee et al., 2013). Boelee et al. (2013) and Boelee et al. (2012) stated that low uptake of nutrients during winter may be one of the main limitations for microalgae remediation systems in temperate regions. However this does not take into account the fact that Bran Sands STW has access to large amounts of waste heat and has a great deal of artificial lighting around the site. Thus further work would be required to determine whether the reduced light levels in winter would affect nutrient uptake. As ammonia damages the PSII system in microalgae (Drath et al., 2008), not being subjected to constant illumination may increase growth as time is given for photosystems to repair. Additionally certain studies have found that nutrients are consumed more rapidly through heterotrophic growth than autotrophic growth (Bohutskyi et al. (2014) and references within), so it may be an option to remove the need for lighting altogether.

There is also the fact that growth trials carried out in this study have been done under (mostly) aseptic conditions. If NWL implemented the bioremediation system, work would have to be carried out to determine the impact of growing the microalgae on unsterile ADL in unsterile conditions. There is the chance that predators and pathogens will damage growth rates. However many sources

state that bacteria can have growth promoting properties (de-Bashan et al. (2004) and references within). Additionally the photos shown in Section 3.9.4 appear to show that bacteria populations are kept under control as long as suitable trace elements are supplemented.

Further work could also be carried out to monitor how efficiently microalgae strip ammonium and phosphate from the ADL with cultures of varying densities and growth rates, as well as analysis of the N: P uptake ratios and uptake of other undesirable compounds (such as heavy metals).

Finally, once the boundaries of growth rate and nutrient uptake were better understood, further research could be carried out into suitable byproducts that could be extracted from the microalgae biomass (Mata et al., 2010, Spolaore et al., 2006), microalgae cultivation systems (photobioreactors and open ponds) (Cai et al., 2013a, Leite et al., 2013) as well as harvesting techniques (Leite et al., 2013, Boelee et al., 2013, Brennan and Owende, 2010).

Overall the data collected during this research project has shown that microalgae remediation of ADL is potentially a feasible option for Bran Sands STW, as long as measures are taken to ensure the pH, trace element and dilution conditions are suitable. The excess heat, light and CO_2 available at NWL sites make it a much stronger candidate for microalgae bioremediation than many other waste processing sites at temperate latitudes. Therefore it is the author's hope that this study will act as a foundation for future work that will go on to assess the economic viability of microalgae bioremediation, and maybe lead to the construction of a fully working bioremediation system in the not too distant future.

Appendix A: Methods Evaluation

A1: Wavelength Scans

A1i: Objective

1) Decide on appropriate method to measure microalgae growth
2) Decide on appropriate wavelength to measure microalgae in turbid solution

A1ii: Overview

Two key parameters that had to be measured in this study were nutrient concentrations and the growth of the microalgae. Thus a great deal of time at the beginning of the study determining the best methods to carry out these tasks. A common method used to measure microalgae growth is optical density measurements, i.e. measuring the absorbance of the culture with a spectrophotometer. Initially it was unclear whether growing the microalgae in a turbid solution would affect the reliability of OD measurements. This is a common problem when attempting to quantify the amount of phytoplankton present in turbid rivers and lakes, or the growth inhibition caused by a toxic particle. Different studies use different methods to tackle the problem of quantifying algae growth in turbid solutions. Some studies relied on cell counts (Lund et al., 1958); e.g. Søballe and Kimmel (1987) used Secchi disk readings and algae cell counts to create a non-algal turbidity index. Alternatively Padisák and Dokulil (1994) calculated the biomass of phytoplankton in lake samples by performing cell counts, measuring the dimensions of the algae and multiplying these factors together. Wang (1974) measured the weight of the dry suspended solids, then washed the samples with hydrochloric acid to remove inorganic $CaCO_3$ and calculated the difference between these measurements to be the weight of the planktonic biomass. Many studies looked at measuring chlorophyll *a.* May et al. (2003) used chlorophyll *a* data from the US Geological Survey (USGS) in San Francisco Bay to model effect of turbidity on phytoplankton blooms, as well as a proxy for biomass (units: mg Chl *a* / m^3). Wei et al. (2010) were investigating the aquatic ecosystem safety of silica nanoparticles by exposing *Scenedesmus obliquus* to SiO_2 nanoparticles and bulk particles. The growth inhibition was measured monitoring the chlorophyll and carotenoid content of the cells. Dokulil (1994) compared chlorophyll *a* concentrations to total suspended solids to investigate factors affecting phytoplankton productivity in turbid systems. Allende et al. (2009) measured chlorophyll *a,* pigment extracts, took counts of micro- and nano- plankton using an inverted microscope, counted picoplankton by fluorescence given off by photosynthetic pigments and measured photosynthetic rates as a function of irradiance by the C^{14} method (as described by Steeman-Nielsen (1952)). Aruoja et al. (2009) created custom made two chamber growth flasks, one containing the culture and the other turbid solution to quantify the effect of shading by particles. Protein assays were also considered as a method to measure microalgal biomass.

Overall it appears that chl-*a* is currently the favoured method of measuring phytoplankton in turbid environments, while pigment analysis and cell counts were typically used when investigating species variation (which is not applicable to this project). Unfortunately, chlorophyll *a* measurements typically take about 24 hours and have to be measured using expensive quartz cuvettes (as the

solvents used to extract the chlorophyll would melt the plastic cuvettes usually used in spectrophotometers). This is not an issue if a one off experiment is being carried out, however when taking daily measurements of multiple flasks (up to 24) this becomes quite a time-consuming cumbersome technique that can be avoided if the OD of the digestate is relatively stable and reproducible. An alternative method that could have been used was in vivo fluorescence (Kalaji et al., 2014), however to the best knowledge of the author this method was not regularly carried out in the School of Biological and Biomedical Sciences, so the equipment was not available to conduct these measurements. For these reasons it was decided that if the OD of the ADL was stable, microalgae growth would be measured by taking absorbance measurements with a spectrophotometer.

A1iii: Anaerobic digestate liquor dilutions

A full wavelength scan was carried out to test whether the ADL had a constant spectral signal.

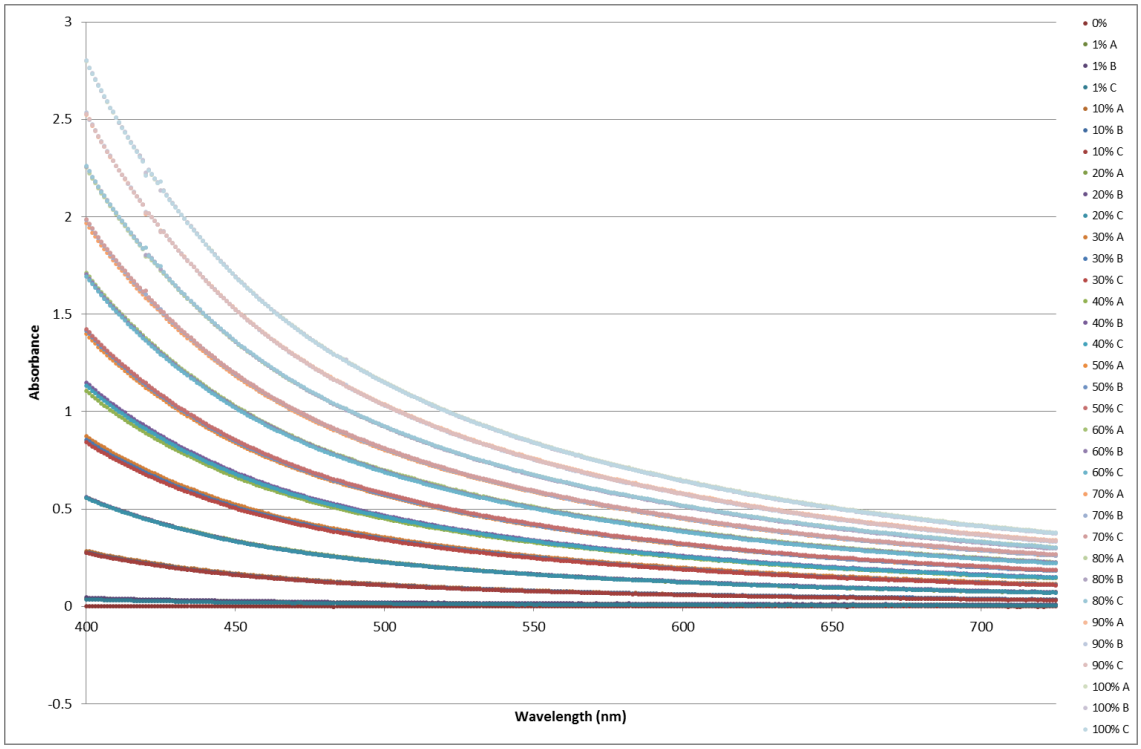

Figure 45: Full wavelength scans of dilutions of AD2901 between 400 nm and 725 nm at 1 nm intervals. Each dilution is tested in triplicate (A, B and C) and the number in the names on the legend refer to the percentage of the sample that is ADL (e.g. "60% A" is a solution containing 60% ADL: 40% deionized water). "0%" shows the spectral signal of deionised water (no absorbance as the spectrophotometer was blanked with deionized water).

Figure 45 shows that the spectral signal of the anaerobic digestate follows a very distinct pattern that appears to be constant and reproducible in multiple tests. This signal is reproduced in AD 1 (collected in November) and AD1703 (collected in March) (see Appendix B5), although the readings show slightly more variation due to a greater % of suspended solids in those samples. This lack of variation in the OD signal of the ADL meant that it was possible to consider OD measurements as a way to monitor microalgae growth.

To determine the impact of particle size on the spectral signal of the ADL, wavelength scans were carried out on unfiltered and filtered ADL.

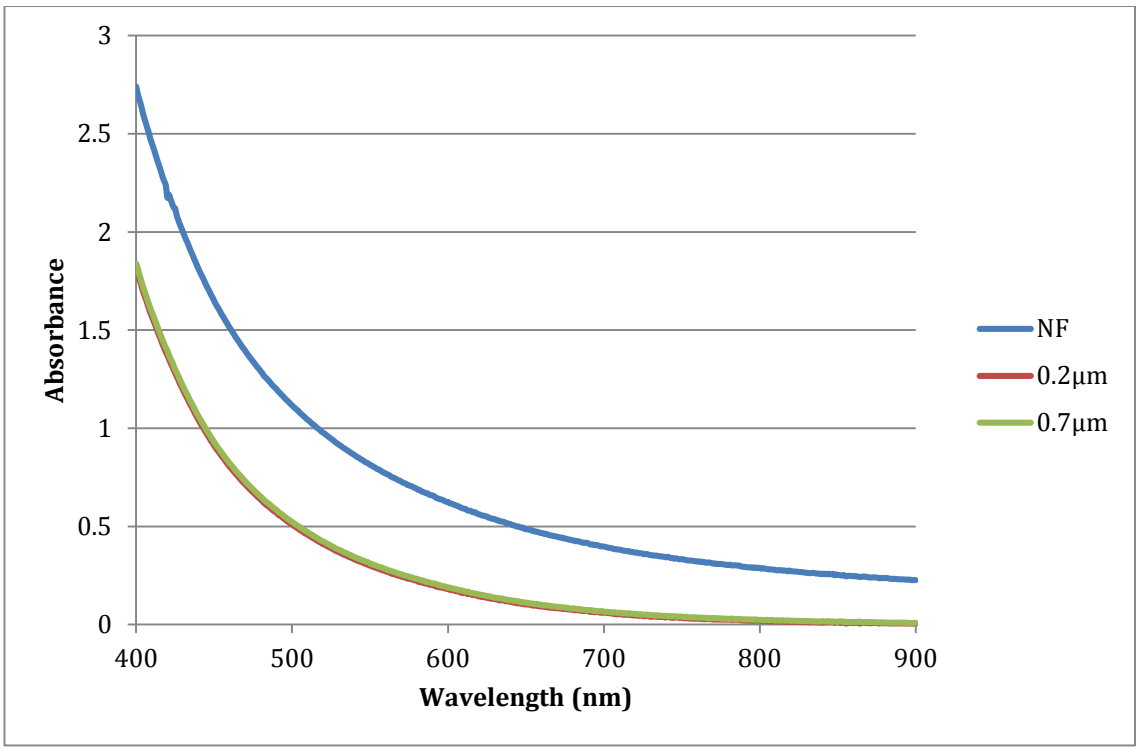

Figure 46: Wavelength scans of filtered and unfiltered samples of AD2901, between 400 nm and 900 nm at 1nm intervals, carried out on the 20/02/2014. "NF" = unfiltered digestate, "0.2µm" = digestate filtered through a Whatman GD/X filter, "0.7µm" = digestate filtered through a Whatman GF/F filter.

Figure 46 shows that suspended solids > 0.7 µm do increase the absorbance of the ADL, while those between 0.2 µm – 0.7 µm have a negligible effect on the absorbance of the ADL between 400-900 nm. The spectral signal of the digestate appears to be one of exponential decrease and the difference between the signal of unfiltered digestate and digestate filtered to 0.7 µm appears to consistently decrease as wavelength increases.

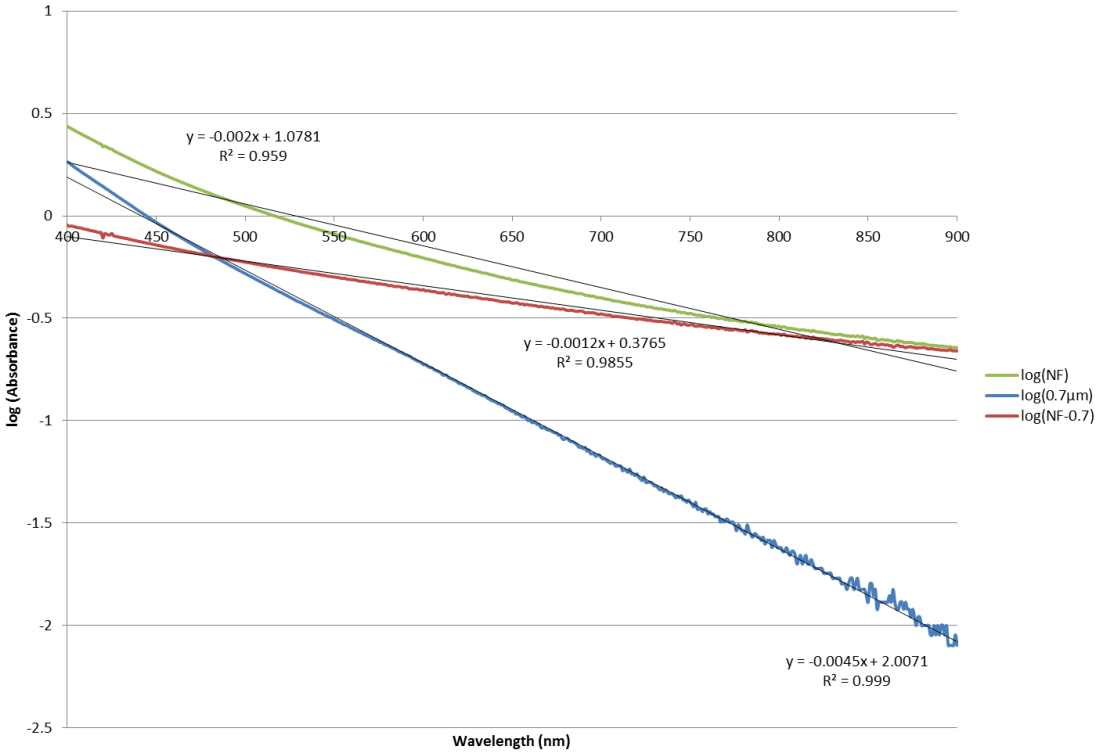

Figure 47: Logarithms of the absorbance of unfiltered ADL (log(NF)), ADL filtered to 0.7 µm (log(0.7µm)) and the difference between unfiltered and filtered ADL (log(NF-0.7)). Trend lines have been added using Microsoft Excel, as well as the equation and of the trend line and R^2 value (also calculated by Excel).

Figure 47 shows that the absorbance signal AD2901 filtered to 0.7 µm is essentially a decreasing exponential signal. A linear trend line with the equation y = -0.0045x + 2.0071 was calculated using the method of least squares, with an R^2 value of 0.999. The unfiltered ADL fits slightly less perfectly into the exponential decay model, with an R^2 of 0.959, while the difference between the unfiltered and filtered ADL can be characterised as an exponential decrease, with an R^2 value of 0.9855. Overall these scans illustrate that the spectral signal of anaerobic ADL is consistent, quantifiable and can typically be modelled as exponentially decreasing as wavelength increases.

If the ADL had been highly variable, when an OD measurement was taken of microalgae growing in a dilution of ADL, it would be difficult to distinguish what proportion of the absorbance was caused by the ADL compared to the microalgae culture. However, as the OD measurements appeared relatively constant and predictable, it meant that a set amount of the overall absorbance signal could be assumed to be a reliable, unchanging signal from the ADL, such that the rest of the signal could be assumed to be caused by the microalgae. This lack of variability allowed the use of OD measurements to monitor the growth of microalgae, even in a turbid solution.

A1iv: Water vs. Bold's Basal Medium – Spectral Analysis

Microalgae are typically cultured in growth medium, whilst deionised water would be used in the blanks used in spectrophotometric measurements, as well as in dilutions of ADL. To test whether the spectral properties of water and Bold's Basal Medium (the growth medium used throughout this series of experiments) were similar, wavelength scans were carried out on both.

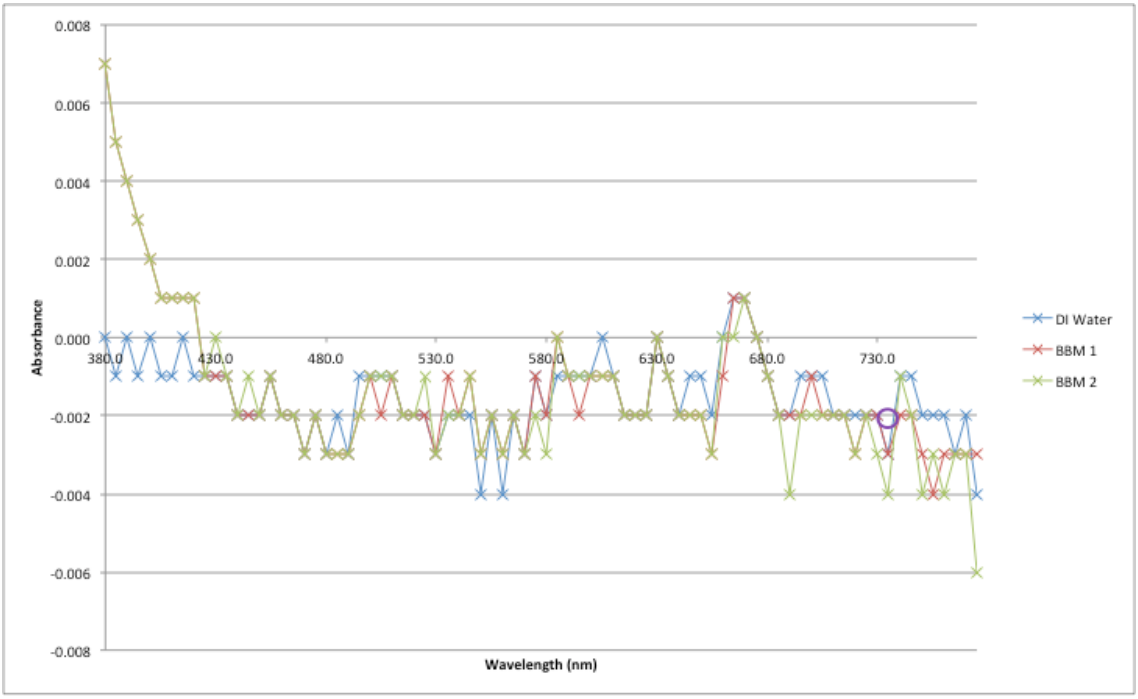

Figure 48: Wavelength scan of deionised water and two samples of BBM (blanked with DI water). The small purple circle shows the absorbance signals of DI Water, BBM 1 and BBM 2 at 725nm.

The spectral signals of deionised water compared to Bold's Basal Medium (when blanked with DI water) are shown in Figure 48**.** The variation in the signal of the DI water shows that the spectrophotometer had an error of approximately ± 0.002-0.004. When this small error is taken into account, it can be seen that the difference between the absorbance signals of DI water and BBM is negligible between 390 nm and 750 nm.

A1v: *Scenedesmus obliquus* Spectral Analysis

Full wavelength scans provide useful information, however when attempting to monitor the growth of cultures on a daily basis a reading at a single wavelength is all that is necessary. This wavelength should ideally be one where the impact of chlorophyll peaks is minimal. Therefore a wavelength scan was carried out on 1ml of *Scenedesmus obliquus,* to determine a suitable wavelength to measure the culture.

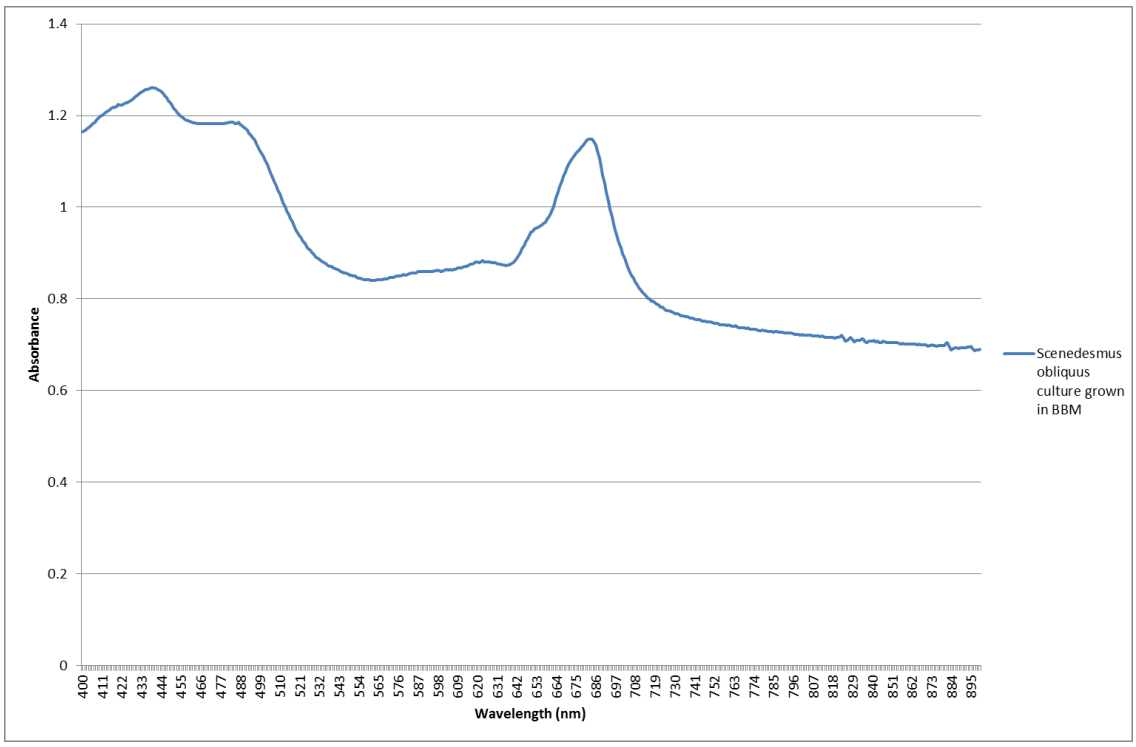

Figure 49: Wavelength scan of 1ml of undiluted culture of *Scenedesmus obliquus*, growing in BBM, between 400 nm and 900 nm at 1 nm intervals.

Distinct peaks can be seen in Figure 49 at approximately 680 nm and between 400 nm and 550 nm. This is due to pigments within the cell absorbing these wavelengths. The main porphyrin pigments present in *S. obliquus* are chlorophyll *a* and *b* and their allomers. The main carotenoid pigments are neoxanthin, loroxanthin, violaxanthin and lutein. β-carotene is present under certain culturing conditions (Wiltshire et al., 2000). The spectral signal of different pigments can be seen in Figure 50.

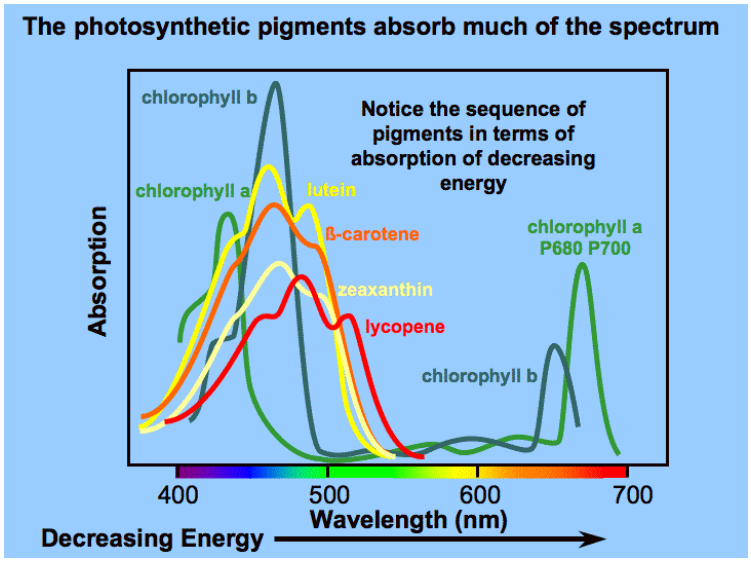

Figure 50: Graphic illustrating typical spectral absorbance of chlorophyll *a*, chlorophyll *b*, lutein, β-carotene, zeaxanthin and lycopene (obtained from Koning (1994)).

A1vi: Microalgae and anaerobic digestate liquor

To double check that the signal caused by the ADL could be reliably predicted when in a microalgae solution, wavelength scans were carried out on ADL, microalgae and ADL and microalgae (Figure 51.

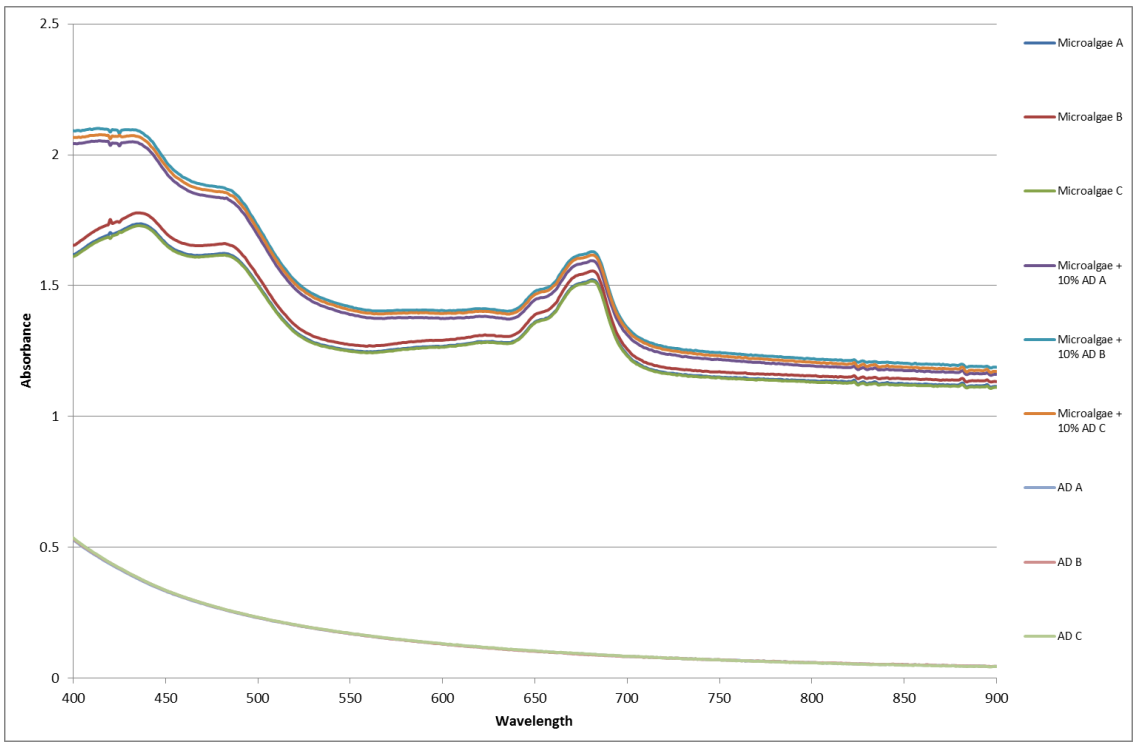

Figure 51: Wavelength scan of microalgae, microalgae & ADL and ADL. The scan is taken between 400 nm and 900 nm at 1 nm intervals. Microalgae = 0.5ml of *S. obliquus* culture diluted with 0.5ml of DI water (in triplicate); Microalgae + 10 % AD = 0.5ml of *S. obliquus* diluted with 0.4ml of DI water and 0.1ml of ADL (in triplicate); and AD = 0.1ml of ADL diluted with 0.9ml of DI water.

When the signal of the microalgae and the signal of the ADL (measured separately) were added together – the results were near identical to a solution containing both microalgae and ADL (Figure 52). The final decision to be made was which wavelength to take OD measurements at. The ideal wavelength would have little absorption from both the microalgae and the ADL. Additionally, the wavelength had to be within the window of 340-750 nm to ensure that the cuvettes allowed the reading to be taken accurately. With respect to the ADL, the higher the wavelength, the lower the absorbance. With respect to the microalgae, the pigment peaks needed to be avoided. Although it could be possible to measure the digestate in the window between the two peaks (500 nm – 620 nm), the lowest absorbance values were recorded above 700 nm. 725 nm was chosen as the wavelength to measure the cultures. It is a high wavelength so the digestate has a low absorbance and it only catches the tail end of the chlorophyll peak at 680 nm. However it is low enough to comfortably fit in the 340 nm – 750 nm window for the cuvettes to work accurately.

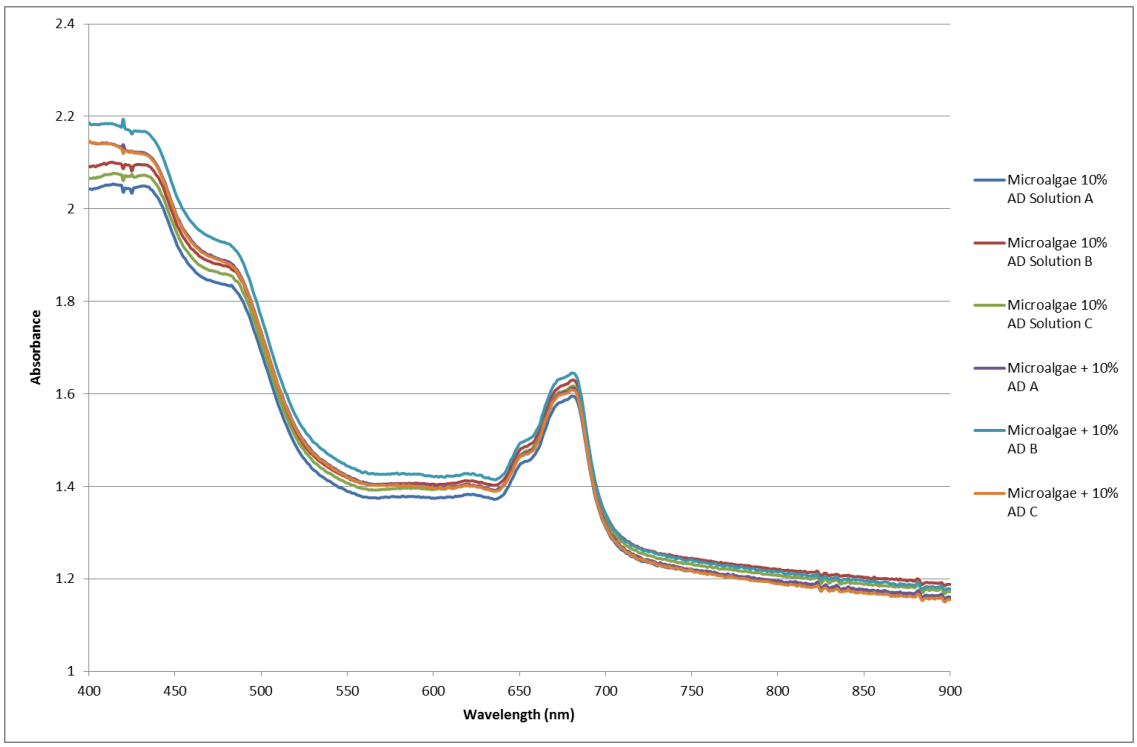

Figure 52: Wavelength scans of microalgae & ADL vs. microalgae + ADL. "Microalgae 10 % AD Solution" refers to a solution composed of 0.1 ml of ADL, 0.5ml of microalgae and 0.4 ml of DI water. "Microalgae + 10 % AD" is the separate signals of two different solutions, added together. The first solution was 0.5 ml of microalgae and 0.5 ml of DI water; the second solution was 0.1 ml AD and 0.9 ml of DI water. Wavelength scans were carried out on these solutions between 400 nm and 900 nm at 1 nm intervals.

A2: Optimisation and validation of nutrient analysis methods

A2i: Objective

1) Ensure methods used to measure nutrients at Bran Sands and the University were comparable
2) Test whether autoclaving and storage affected the nutrient concentrations of the ADL.

A2ii: Hach Lange vs. Ion Chromatography 1

At Bran Sands STW a Hach Lange spectrophotometer was used to measure the nutrient concentrations of the ADL. However, there was no Hach Lange spectrophotometer on the University site, so to test the samples with the same method they would have to be stored and then driven to Bran Sands and tested en masse periodically, or sent to the Howie Lab in batches and tested by Northumbrian Water technicians. The other option was to carry out IC analysis on site at the University. This option was chosen as it simultaneously produced extra data about other anions and cations and reduced storage time as samples could be dropped off the same day that they were taken. However, there was a large difference observed in the nutrient concentrations measured in the sample taken onsite at Bran Sands using the Hach Lange spectrophotometer and the nutrients measured with ion chromatography.

Table 13: AD1 nutrient concentrations measured using IC analysis and a Hach Lange spectrophotometer. AD 1 (Nov) was collected on the 21/11/13, stored at 4^0C until the 27/11/13, then filtered through a 1.2µm filter and tested using a Hach Lange spectrophotometer. AD 1 (Jan) is the same batch of AD liquor; however it has been subjected to autoclaving, 6 weeks of storage and filtration through a 0.2µm filter and tested with ion chromatography analysis in January 2014.

	Concentration (mg/l)	
	AD 1 (Nov)	**AD 1 (Jan)**
NH_4^+	3750	1059
NO_3-N	11.8	1
PO_4-P	356	111

Table 13 shows that the concentrations measured by the Hach Lange spectrophotometer (methods discussed in Section 2.2.1) shown are larger than those measured with IC analysis. The concentrations of the ammonium and phosphate (as P) are approximately 3 times larger in the Hach Lange sample, compared to the IC sample. The nitrate concentration is 11 times greater in the 1st sample compared to the second. There were a large number of possible reasons for the differences seen in the nutrient concentrations.

Firstly there was the chance that the nutrients had changed dramatically over the 8 weeks that AD1 was stored. The second was that autoclaving the ADL had drastically altered the nutrient concentrations. For example, Franchino et al. (2013) state that autoclaving reduced the ammonium concentration in agro-zootechnical digestate by 60% (while the ammonium concentrations in the digestate in AD1 appeared to decrease by 72%).

Alternatively, there may be discrepancies in the measuring capabilities of the spectrophotometer and the ion chromatography unit, so although the nutrient concentrations had not changed, the two machines simply displayed different results (important to resolve if NWL was to take this study further). Additionally, the calculated phosphate concentration of AD 1 (Nov) (Table 1) is inaccurate as it was out of the range required for the instrument to reliably calculate the concentration (at 50 times dilution the concentration of phosphate measured was 7.12 mg/l, outside of the reliable range of 0.5-5.0 mg/l – see Appendix B4). Due to time constraints on the day (each phosphate measurement takes over an hour and a half) it was not possible to re-dilute and retest the sample.
Another possible reason was the different pore sizes of the filter papers used to filter the two samples. Both samples were filtered using glass microfiber filter papers, however the samples tested at Bran Sands were only filtered to 1.2 µm, while the second sample had to be filtered to 0.2 µm to have IC analysis carried out. It was possible that the particles (0.2-1.2 µm in diameter) contained high concentrations of phosphate, and by removing these particles the overall concentration of the ADL was reduced. Finally the differences could have been due to human error. As the technical staff that carried out the IC analysis are specifically trained to use the instrument, it seemed more likely that the author had made a mistake.

A2iii: Hach Lange vs. Ion Chromatography 2

To resolve the cause of this discrepancy, the autoclaved sample tested with IC (filtered to 0.2 µm) was retested using the colorimetric method. Additionally a new sample was collected, filtered with a 1.2 µm filter and measured using the colorimetric method; then returned to the Durham University site, autoclaved, filtered to 0.2 µm and measured using IC analysis.

Table 14: Concentrations of an autoclaved ADL sample filtered and tested in different ways. "AD 0 (1.2µm) (HL)" is the unautoclaved sample, filtered to 1.2µm and tested using a Hach Lange spectrophotometer in November 2013. "AD 1 (0.2µm) (IC)" is the sample filtered to 0.2µm and tested with IC analysis in January 2014. "AD 1 (0.2µm) (HL)" is the sample which had IC analysis carried out on it, retested using a Hach Lange spectrophotometer in January 2014. "AD 1 (1.2µ) (HL)" is from the same bottle as the other two; however it had previously been unfiltered. In this experiment it was filtered to 1.2µm and then tested with a Hach Lange spectrophotometer in January 2014.

	Concentration (mg/l)			
	AD 1 (Nov) (1.2 µm) (HL)	**AD 1 (Jan) (0.2 µm) (IC)**	**AD 1 (Jan) (0.2 µm) (HL)**	**AD 1 (Jan) (1.2 µm) (HL)**
PO_4-P	356	111.48	120	132
NH_4^+	3750	1059.5	1175	1096.5

The results in Table 14 show that both the phosphate and ammonium concentrations recorded for AD 1 (Jan) are relatively similar, independent of the measurement method (IC or colorimetric testing) and filter pore size. This suggests that the values obtained from colorimetric testing and IC

analyses are comparable. The largest difference is between the nutrient concentrations measured in November, versus the concentrations measured in the same ADL in January. This implies that the difference in values was due to sampling error when the first time colorimetric testing was carried out, or the properties of the ADL changed over the storage period/when autoclaved. Additionally the data shows that it does not make much difference to the nutrient concentrations if the ADL is filtered to 0.2 µm or 1.2 µm. Either the solid particles do not contain a significantly greater portion of the available nitrogen and phosphorus in the ADL, or the nitrogen and phosphorus locked up in the solid fraction of the ADL cannot be measured using either technique. A new sample (AD2901) was also collected, tested using the Hach Lange spectrophotometer, then returned to Durham University, autoclaved, filtered and tested using IC analysis.

Table 15: Phosphate and ammonium concentrations of AD2901, tested using a colorimetric method (pre-autoclave) and ion chromatography analysis (post-autoclave).

	AD2901 (1.2 µm) (HL)	**AD2901 (0.2 µm) (IC) (Autoclaved)**	**Decrease (mg/l)**	**Decrease (%)**
PO_4-P	123	101.4	21	17.39 %
NH_4^+	1938	1472	466	24.05 %

Table 15 shows that the phosphate and ammonium concentrations decreased in AD2901 once it had been stored, autoclaved and measured with IC. Phosphate decreased by 17% and ammonium by 24%. With the confirmation that IC analysis and colorimetric testing are comparable (Table 2), it is possible to infer from the data in Table 3 that the large decrease in nutrients is either caused by storage or autoclaving. Despite the large total decrease in ammonium and phosphate (24 % and 17 % respectively), Table 4 shows that the N:P ratio is less affected, only decreasing by 8 %.

Table 16: Nitrogen: Phosphorus ratio of AD2901, measured using a Hach Lange spectrophotometer before autoclaving and using ion chromatography analysis after autoclaving. Note that the N: P ratio is calculated as the ratio of concentrations between PO_4-P and NH_4^+-N, not NH_4^+.

	AD29/01 (1.2µm) (HL)	**AD29/01 (0.2µm) (IC) (Autoclaved)**	**Change (mg/l)**	**Change (%)**
N:P	12.23	11.27	-0.96	-8 %

As the experiments in this thesis were based on growing microalgae on dilutions of ADL, the nutrients decreasing whilst maintaining similar ratios to each other was favorable, compared to the situation of the nutrients decreasing and changing relative to each other. Further data collected during these experiments can be found in Appendix B7.

With regards to storage, AD 1 was stored at 4 °C for a total of 12 weeks, four weeks before autoclaving, then IC analysis was carried out 6 weeks after autoclaving and the second round of

colorimetric testing was carried out in week 12. In the four weeks before autoclaving, bacteria were still present so there is the chance that microbes could have biologically altered the nutrient composition of the ADL. However most of the bacteria present in the anaerobic digestate would be mesophilic or thermophilic, so it is unlikely that bacteria would have altered the nutrient concentrations that much when the ADL was stored at 4 °C. Additionally it is unusual for covered ADL to lose large quantities of nitrogen to the atmosphere during storage, especially if the depth to surface area ratio is high (Whelan et al., 2010), which would be the case in the closed bottles the ADL was stored in.

A2iv: Temporal nutrient stability measurements

The N and P concentrations could have been reduced chemically over the storage period. To test whether autoclaved ADL nutrient concentrations remained stable whilst stored at 4 °C, 3 samples of AD1703 were measured 3-4 times per week between the 09/04/14-24/04/14. The sample was collected and autoclaved in March and was called AD1703-2 (the 2 referring to the fact it was autoclaved in the second of 3 bottles).

This amounted to 10 measurements in total, however the cation data for 17032-B is calculated from 9 measurements due to one set of values being an anomaly. Table 17a shows the mean values, Table 17b displays the standard deviations and Table 17c shows the relative standard deviations. Table 17d shows the N:P ratio of the samples are similar (between 9.7-10.2) with a small standard deviation of 0.7-1.0. The main objective of this experiment was to determine whether N and P values remained fairly constant over time. As a large amount of time and sample volume are required to accurately measure each nutrient, these experiments simply aimed to have the correct dilutions for the phosphate and ammonium measurements and approximate the values of the other nutrients from non-ideal dilutions. Due to high ammonium and phosphate concentrations, the dilutions required to accurately measure them are 1000 times and 25 times respectively. This meant that it was not possible to measure nitrite, nitrate, bromide, magnesium and calcium, due to their low concentrations. However every other ion measured appeared to remain relatively stable over the 3-week period, except for sulphate, which exhibited a clear downward trend over time.

Tables 17a-d show that ammonium and phosphate concentrations do not change dramatically over a three week period, neither does the N:P ratio. Therefore, it can be concluded the most likely reasons for the nutrient discrepancies seen in AD1 between November and January, and the difference between AD2901 tested with a spectrophotometer and ion chromatography are partially due to human error during the measuring process and partially due to autoclaving reducing the nutrient concentrations.

Table 17a, b, c and d: Mean ion concentrations, Standard deviation, Relative Standard Deviation and N:P ratio of AD1703 flasks over 3 weeks respectively. n = 10, except for the ammonium, sodium and potassium for 17032-B, where n=9.

Mean Values							
	Phosphate	**Fluoride**	**Chloride**	**Sulphate**	**Ammonium**	**Sodium**	**Potassium**
17032-A	115.41	17.67	169.91	15.69	1433.65	72.81	138.61
17032-B	114.95	17.64	168.29	15.80	1533.06	77.82	134.08
17032-C	115.59	17.58	168.38	15.65	1448.05	72.26	137.35

Population Standard Deviation (σ)							
	Phosphate	**Fluoride**	**Chloride**	**Sulphate**	**Ammonium**	**Sodium**	**Potassium**
17032-A	9.85	2.18	7.67	6.11	77.35	22.91	45.90
17032-B	10.99	2.13	7.80	6.16	104.57	20.02	37.11
17032-C	10.59	1.71	7.82	5.93	81.68	18.97	42.04

Relative (Population) Standard Deviation (% RSD)							
	Phosphate	**Fluoride**	**Chloride**	**Sulphate**	**Ammonium**	**Sodium**	**Potassium**
17032-A	9%	12%	5%	39%	5%	31%	33%
17032-B	10%	12%	5%	39%	7%	26%	28%
17032-C	9%	10%	5%	38%	6%	26%	31%

N:P			
	Mean	**Σ**	**%RSD**
17032-A	9.7	1.0	11%
17032-B	10.2	1.1	11%
17032-C	9.8	0.7	8%

Appendix B: Supplementary Results

B1: Calibrated standards ion chromatography machine

The anion and cation calibration standards used in the ion chromatography machine are shown in Table 18 and Table 19. Methods discussed in Section 2.3.

Table 18: Anion calibration standards (mg/l). std = standard.

Ion	std 1	std 2	std 3	std 4	std 5	std 6
Fluoride	0.5	1	2	3	4	5
Nitrite as N	0.5	1	2	3	4	5
Bromide	0.5	1	2	3	4	5
Phosphate as P	0.5	1	2	3	4	5
Nitrate as N	1	2	4	6	8	10
Sulphate as S	1	2	4	6	8	10
Chloride	4	8	16	24	32	40

Table 19: Cation calibration standards (mg/l). std = standard.

Ion	std 1	std 2	std 3	std 4	std 5	std 6
Ammonium	0.5	1	2	3	4	5
Potassium	0.5	1	2	3	4	5
Magnesium	1.5	3	6	9	12	15
Sodium	3	6	12	18	24	30
Calcium	5	10	20	30	40	50

B2: Nutrient concentrations of Prepared Growth Mediums

Table 20: Concentrations of chemicals in stock solutions used to make the growth mediums Bold's basal medium and Bold's basal medium – N and P. The concentrations are calculated from the mass of the component, the volume of the stock solution and the volume added to the final growth medium. * = used in BBM, not BBM (-NP). ** = used in BBM (-NP), not BBM.

Stock solution	**Volume (ml)**	**Component**	**Mass (mg)**	**Concentration (mg/l)**	**Volume in final 1l of BBM (ml)**	**Final Concentration (mg/l)**
Sodium nitrate*	400	$NaNO_3$	10000	25000.0	10	250
Magnesium sulfate • $7H_2O$	400	$MgSO_4.7H_2O$	3000	7500.0	10	75
Sodium chloride	400	NaCl	1000	2500.0	10	25
Potassium phosphate dibasic trihydrate *	400	$K_2HPO_4.3H_2O$	3000	7500.0	10	75
Potassium phosphate monobasic *	400	KH_2PO_4	7000	17500.0	10	175
Calcium chloride dihydrate	400	$CaCl_2.2H_2O$	1000	2500.0	10	25
Trace Element	1000	$ZnSO_4.7H_2O$	8820	8820.0	1	8.82
	1000	$MnCl_2.4H_2O$	1440	1440.0	1	1.44
	1000	MoO_3	710	710.0	1	0.71
	1000	$CuSO_4.5H_2O$	1570	1570.0	1	1.57
	1000	$Co(NO_3)2.6H_2O$	490	490.0	1	0.49
Boron	100	H_3BO_3	1140	11400.0	1	11.4
EDTA	100	EDTA Na2	5000	50000.0	1	50
	100	KOH	3100	31000.0	1	31
Iron	1000	$FeSO_4.7H_2O$	4980	4980.0	1	4.98
	1000	H_2SO_4	(see Table 4)			
NaCl + KCl**	500	NaCl	8600	17200.0	10	172
	500	KCl	8000	16000.0	10	160
HEPES	250	$C_8H_{18}N_2O_4S$	238.3	95.3	50	4766

Table 21: Concentrations of chemicals in stock solutions used to make the growth mediums Bold's basal medium and Bold's basal medium – N and P. The concentrations are calculated from the volume of the component, the volume of the stock solution and the volume added to the final growth medium.

Stock solution	Volume (ml)	Component	Concentration of Component (mM)	Volume (ml)	Concentration (mM)	Volume in final 1l of BBM (ml)	Final Concentration (mM)
HEPES	250	$C_8H_{18}N_2O_4S$			400.0	50	20
Sulphuric acid	1000	H_2SO4	18000	1	18	1	0.018

Table 20 and Table 21 show the concentrations of different chemicals in the BBM and BBM (-NP) growth media used in Experiment 8.

B3: Photon flux density

The photon flux density in the growth room was measured every second over 33 minutes on the 11/08/2014. This is shown in Figure 53. The meter was placed at the height of the flasks in the growth room to ensure that the photon flux recorded was the amount the flasks received, not the flux present at a different height or area of the room.

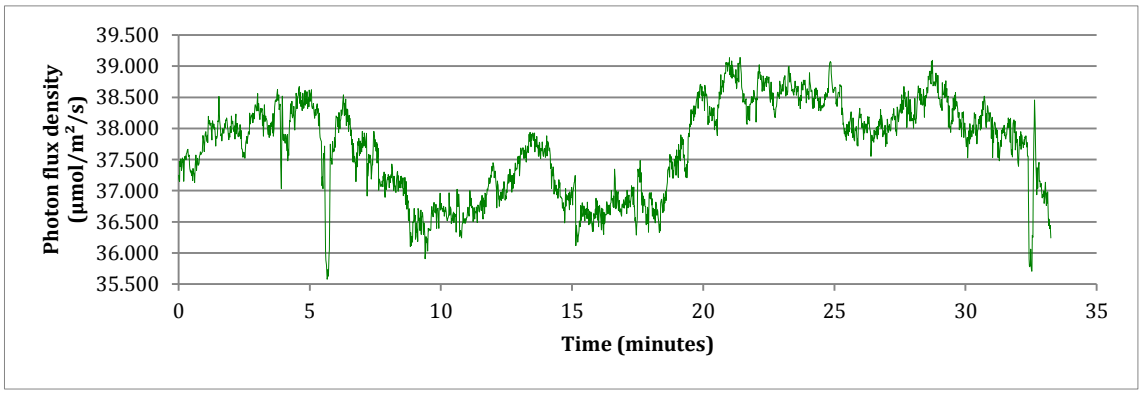

Figure 53: Photon flux density recorded in growth room over a 33 minute period (µmol $m^{-2}s^{-1}$).

The sudden drops (negative spikes) seen on the graph typically appeared as the author entered the culture room. The door being opened likely caused this, allowing light to leave the room and preventing light from being reflected back into the culture room. Once the door had been closed the levels would return to normal. 1997 measurements were taken and the mean light intensity was 37.676 µmol m^{-2} s^{-1}, with a standard deviation of 1.128 µmol m^{-2} s^{-1} (a relative standard deviation of 2.99 %).

B4: Range of Spectrophotometer

Table 22 shows the range of detectable limits for different nutrients measured using a Hach Lange DR3900 spectrophotometer

Table 22: Hach Lange DR3900 spectrophotometer detection limits of PO_4-P, NH_4-N and NH^4 in the concentration ranges relevant to the experiments carried out in this study.

Range:	0.5-5.0mg/l PO_4-P
	2-47mg/l NH_4-N
	2.5-60 mg/l NH_4

B5: AD spectral scans

Figure 54 and Figure 55 show wavelength scans carried out on AD1 and AD1703 respectively.

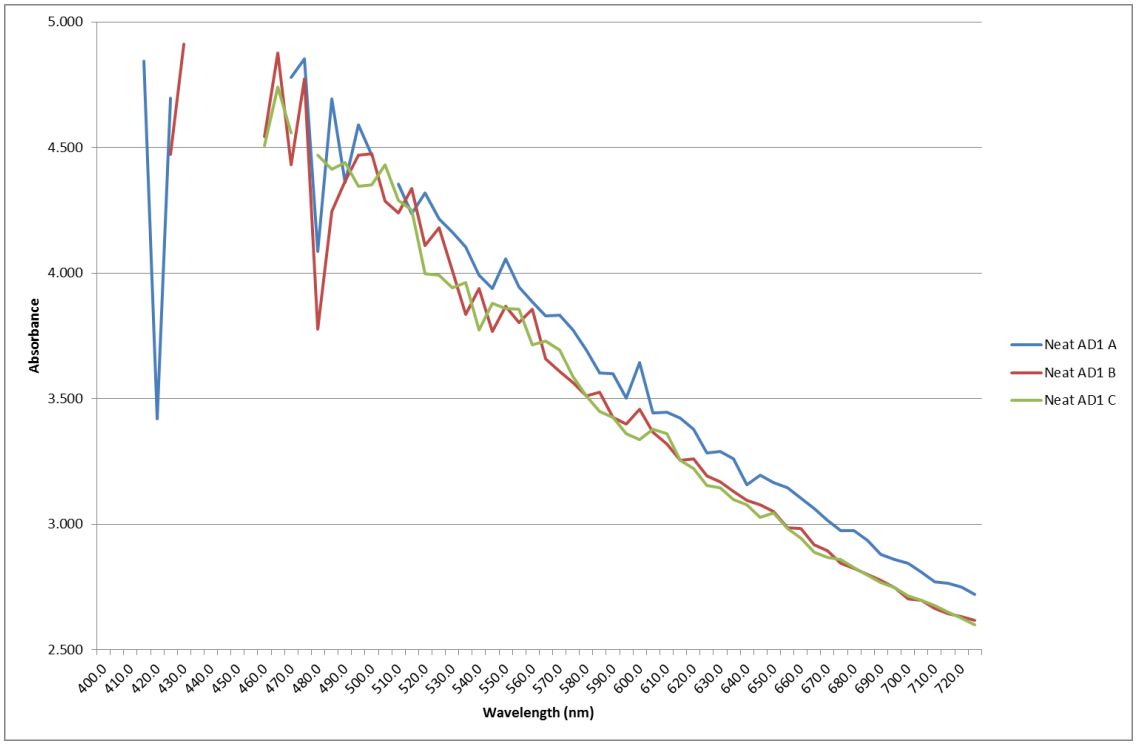

Figure 54: Wavelength scans of three samples of AD1 between 400 nm and 725 nm with measurements taken at 5 nm intervals

Figure 54 shows a wavelength scan carried out on three samples of AD1 on the 27/01/14. These scans show a similar pattern to the data presented in Appendix A1iii, of large readings in the low end of the visible spectrum and absorbance decreasing as wavelength increases. However more variation is seen in this set of scans. This is partly to do with readings being taken at 5 nm intervals instead of 1 nm intervals. AD2901 was also a sample with a particularly low amount of suspended solids, with AD1 and AD1703 containing more suspended material and therefore having higher absorbance signals. Additionally the three AD1 samples tested came from three different bottles of AD1. The original AD1 sample was a 5 l sample in a 5 l container. This 5 l was then decanted into 5 1 l bottles to be autoclaved. There was a chance that despite the sample being shaken before it was poured, gravitational forces would have caused the particle sizes in the different bottles to be different. There

does appear to be variation amongst the bottles, with Neat AD1 A having a slightly higher absorbance (average of + 0.090 compared to the mean of the three samples), which is likely due to differing particle sizes.

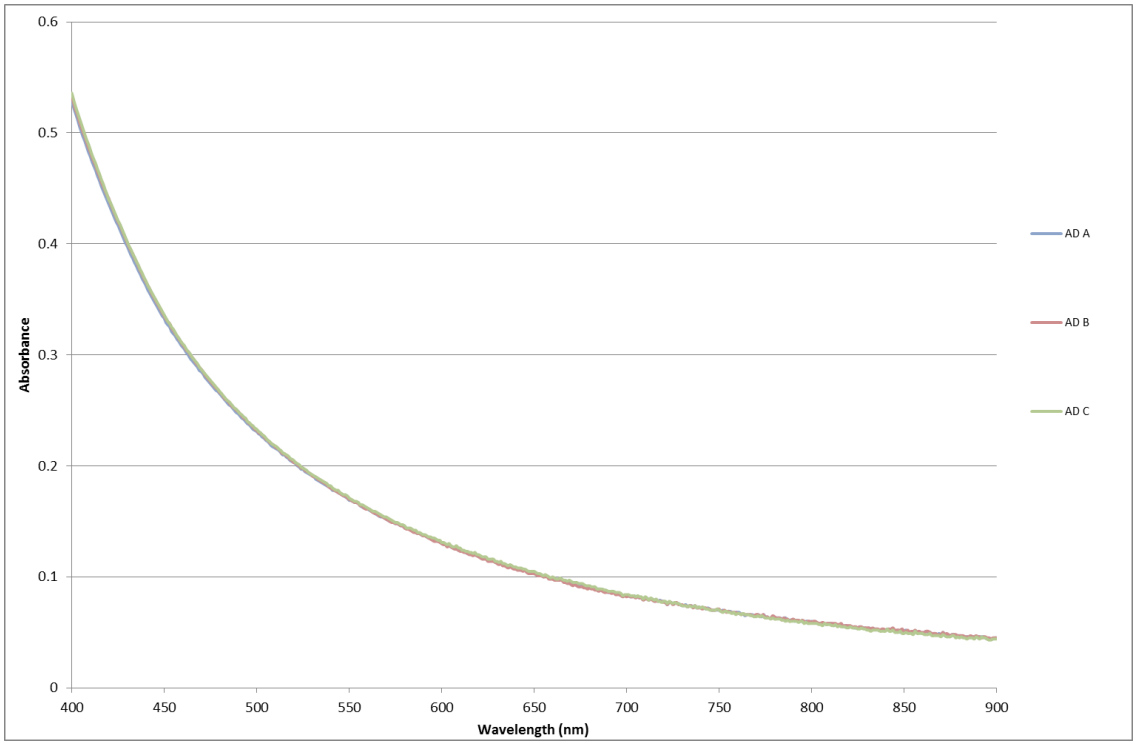

Figure 55: Wavelength scan of a 10 % AD1703 solution (with 90% deionized water) between 400 nm and 900 nm with 1 nm intervals. Scan carried out on the 10/07/2014.

Figure 55 shows that AD1703 has a similar curving decrease in absorbance across the visible spectrum to AD1 and AD2901. The lower absorbance and potentially lower variation is due to the scan being carried out on a 10 % dilution of AD1703, rather than a neat sample. Overall it can be concluded that the absorbance patterns are similar in AD1 and AD1703, as well as AD2901 (Figure 45, Appendix A1).

B6: Molecular weight conversions

Table 23 shows the calculations used to convert between the molecular weight of a nitrogen or phosphorus containing atom and the molecular weight of nitrogen or phosphorus.

Table 23: Conversion chart between nitrogen/phosphorus weight and ionic weight.

Conversions:
NH_3 = NH_3-N x 1.21589
NH_4 = NH_4-N x 1.28786
NO_3 = NO_3-N x 4.42664
PO_4 = PO_4-P x 3.06611997

B7: January Data

B7i: Bran Sands Data

The additional analysis carried out on AD1 at Bran Sands STW laboratory are shown in Table 24 and Table 25.

Table 24: Calculations to determine suspended solids concentration in dilutions of AD1. AD1 diluted by x1 and x50 was filtered and the mass of the filter paper before and after filtration. The difference in mass represents the mass of suspended solids in a specified volume of liquid, which is scaled up to determine the concentration of SS in 1 l.

	Volume filtered (ml)	Dilution	Weight of filter paper (mg)	Weight of filter paper + SS (mg)	Weight of SS on filter (mg)	Concentration (mg/l)
Suspended solids (mg)	10	x 1	351.7	380.6	28.9	2890
	25	x 50	352.2	355.6	3.4	6800

Table 25: Ammonium, COD, nitrate and phosphate concentrations in various dilutions of AD1. The green cells indicate that the measurements are unreliable as the concentration is not in range of the instruments measuring capacity

	Filtered	Dilution	Concentration of dilution (mg/l)	Concentration of Neat Sample (mg/l)
NH_4-N	Y	x25	74.3	1858
	N	x25	61.7	1543
	Y	x50	N/A	N/A
	Y	x100	29.1	2910
COD	Y	x50	302	15100
NO_3-N	Y	x1	11.8	11.8
PO_4-P	Y	x50	7.12	356
Scaled up	**Filtered**	**Dilution**	**Concentration of dilution (mg/l)**	**Concentration of Neat Sample (mg/l)**
NH4	Y	x25	95.7	2392
	N	x25	79.5	1987
	Y	x50	N/A	N/A
	Y	x100	37.5	3748
NO_3	Y	x1	52.2	52.2
PO_4	Y	x50	21.8	1092

B7ii: Ion chromatography

The full data of IC analysis carried out on AD1 and AD2901 are shown in Table 26 and Table 27 respectively.

Table 26: IC analysis of AD1. Nitrite and nitrate values are tested with IC and double-checked with UV detection. The first two nitrate and nitrite values are IC values, the second are UV detection values.

	Autoclaved anaerobic digestate
Fluoride (as F)	9.55
Chloride (as Cl)	188.56
Nitrite (as N)	n.a.
Bromide (as Br)	n.a.
Nitrate (as N)	n.a.
Sulphate (as S)	11.39
Phosphate (as P)	111.48
Nitrite (as N)	n.a.
Nitrate (as N)	0.99
Nitrite (as N)	n.a.
Nitrate (as N)	1.10
Sodium	150.69
Ammonium (as NH_4^+)	1059.50
Potassium	97.26
Magnesium	0.74
Calcium	8.82
Date Analysed	16/01/2014

Table 27: Concentrations of ions present in an autoclaved AD2901 sample (measured using IC analysis).

Ions measured	AD2901 (Autoclaved)
Anions	
Fluoride (as F)	6
Chloride (as Cl)	187.6
Nitrite (as N)	0.12
Bromide (as Br)	1.84
Nitrate (as N)	-
Sulphate (as S)	14.96
Phosphate (as P)	101.4
UV/vis detection	
Nitrite (as N)	0.12
Nitrate (as N)	2.88
Cations	
Sodium	107.36
Ammonium (as NH_4^+)	1472
Potassium	166
Magnesium	5.22
Calcium	5.1
Date Analysed	05/02/2014

The decrease in phosphate and ammonium in AD2901 caused by autoclaving the ADL are shown in Table 28 and Table 29.

Table 28: Phosphate change in AD2901 due to autoclaving

Calculated scaled up Phosphate (PO_4-P) in mg/l				
Dilution	**AD29/01 1.2µm Test kit**	**AD29/01 autoclaved ion chromatography 0.2µm**	**Change (mg/l)**	**Change (%)**
x50	122			
x100	124			
Mean	123	101.4	-21	-17.39%

Table 29: Change in ammonium ion concentration present in AD2901, due to autoclaving

Calculated scaled up Ammonium (NH_4) in mg/l				
Dilution	**AD29/01 0.45µm Test kit**	**AD29/01 autoclaved ion chromatography 0.2µm**	**Change (mg/l)**	**Change (%)**
x50	1945			
x100	1932			
Mean	1938	1472	-466	24.05%

B7iii: Calculation of phosphate and ammonium concentrations from readings taken at multiple dilutions

Table 30 and Table 31 show the how the mean concentrations of ammonium and phosphate in AD1 and AD2901 (quoted in Appendix A2iii) were calculated.

Table 30: Mean phosphate concentrations of AD1 and AD2901. Values in italic are measurements that are inaccurate as concentration was out of range of instrument. Mean was taken of readings that were in range.

Calculated scaled up Phosphate (PO_4-P) in mg/l			
Dilution	**AD1 0.2µm**	**AD1 1.2µm**	**AD29/01 1.2µm**
x20	*113*	*114*	*130*
x50	117	144	122
x100	123	120	124
Mean	120	132	123

Table 31: Mean ammonium concentrations of AD1 and AD2901. Values in italic are measurements that are inaccurate as concentration was out of range of instrument. Mean was taken of readings that were in range.

Calculated scaled up Ammonium (NH_4) in mg/l				
Dilution	**AD1 0.2µm**	**AD1 1.2µm**		**AD29/01 1.2µm**
		Test 1	**Test 2**	
x20	1198	*1404*	*1308*	*1883*
x50	1178	1120	1133	1945
x100	1147	1033	1099	1932
Mean	1175	1077	1116	1938

B8: Ion chromatography results of AD2901 digestate used in Experiment 1

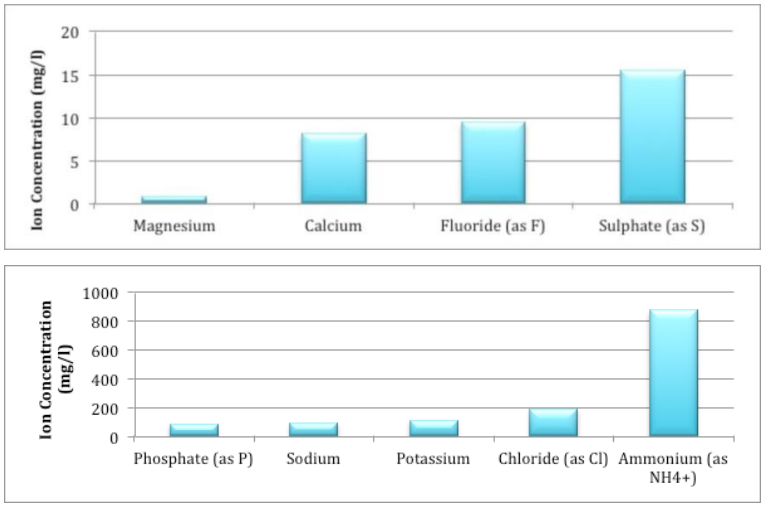

Figure 56: Ion concentrations of AD2901 sample used in Experiment 1, measured using IC analysis.

Figure 56 shows the IC results of the ADL used in Experiment 1. When the ion concentrations are compared to previous measurements of AD2901 (Figure 7, Figure 8 and Figure 9) it can be seen that the results are similar.

B9: Unedited photo of *Scenedesmus obliquus* and solutions in cuvettes

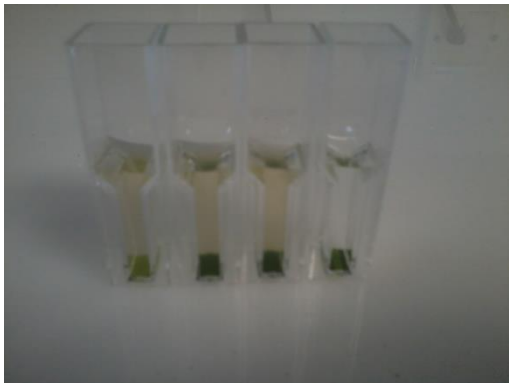

Figure 57: Unedited photo of cuvettes containing *Scenedesmus obliquus* grown on buffered GrM-ad H_2O-AD, GrM-ad BBM-AD, GrM-ad BBM (-NP) AD and GrM-ad BBM-H_2O in Experiment 8.

Settled microalgae cells in cuvettes are shown in Figure 57. The photo used in the main thesis has the exposure increased to better show the difference in colour between the microalgae cells and solutions in each cuvette. This photo is included in the Appendix in its original, unedited form for reference purposes.

Appendix C: Growth Rate

C1: Calculations

Key:

a = OD, a_0 =initial OD, k = growth rate, t = time

Step 1: *Find k*

Find rate of growth using equation:

$$a = a_0 e^{kt}$$

Work out k by rearranging:

$$k = \frac{\ln\left(\frac{a}{a_0}\right)}{t}$$

Step 2: *Work out the area under the curve*

Work out area under growth curve (A_g) using summations of rectangles (A_r) and right-angled triangles (A_t):

$$A_r = a \times b$$

$$A_t = \frac{a \times b}{2}$$

Step 3: *Nutrient calculations*

Find out the area required per ml of nutrient consumption (N_u) by dividing the area under the graph (A_g) by the amount a given nutrient decreased over the recorded period (N_d):

$$N_u = \frac{A_g}{N_d}$$

Step 4: *Work out area required to remediate a certain concentration of nutrient*

Determine what area would be required (N_A) to remediate a given concentration of a nutrient (N_c):

$$N_A = N_u \times N_c$$

Step 5: *Work out the initial OD/time required to remediate a certain concentration of nutrient.*

Find a_0

Choose the time required to remediate the nutrients (t_1). Then, assuming exponential growth throughout, calculate what starting OD (a_0) would be required to remediate a given concentration of nutrient (N_c):

$$N_A = \int_0^{t1} a_0 e^{kt} \, . \, dt$$

$$N_A = a_0 \int_0^{t1} e^{kt} \, . \, dt$$

$$N_A = \left[\frac{a_0}{k} e^{kt} + C\right]_0^{t1}$$

$$N_A = \frac{a_0}{k}(e^{kt1} - 1)$$

$$a_0 = \frac{N_A k}{(e^{kt1} - 1)}$$

Find t_1

Rearrange to find t_1:

$$a_0 = \frac{N_A k}{(e^{kt1} - 1)}$$

$$e^{kt1} = \frac{N_A k}{a_0} + 1$$

$$kt_1 = ln\left(\frac{N_A k}{a_0} + 1\right)$$

$$t_1 = \frac{ln\left(\frac{N_A k}{a_0} + 1\right)}{k}$$

Example with GrM-ad BBM (-NP) AD c

Step 1: *Find k*

a	**a0**	**t1**	**K**
0.6	0.047	6	0.424463676

Step 2: *Calculate the area under the curve*

	OD Data										
Day	**0**	**1**	**2**	**4**	**5**	**6**	**8**	**10**	**11**	**13**	**14**
GrM-ad BBM (-NP)-AD c	0.047	0.062	0.108	0.314	0.413	0.600	1.025	1.200	1.271	1.377	1.481

Dimensions Table											
Rectangle and Triangle a		1	1	2	1	1	2	2	1	2	1
Rectangle b		0.047	0.062	0.108	0.314	0.413	0.600	1.025	1.200	1.271	1.377
Triangle b		0.015	0.046	0.206	0.099	0.187	0.425	0.175	0.071	0.106	0.104
Rectangle area		0.047	0.062	0.216	0.314	0.413	1.200	2.050	1.200	2.542	1.377
Triangle Area		0.008	0.023	0.206	0.050	0.094	0.425	0.175	0.036	0.106	0.052
R + T Area		0.055	0.085	0.422	0.364	0.507	1.625	2.225	1.236	2.648	1.429
Sum of Areas		0.055	0.140	0.562	0.925	1.432	3.057	5.282	6.517	9.165	10.540

Step 3: *Nutrient calculations*

Initial nutrient conc (mg/l)	**Final nutrient conc. (mg/l)**	**Nutrient decrease (mg/l)**	**Area required per mg/l of nutrient decrease**
150.09	89.57	60.52	0.151

Step 4: *Work out area required to remediate a certain concentration of nutrient*

Nutrient concentration to be remediated (mg/l)	**Area required for remediation**
400	60.576

Step 5: *Work out the initial OD/time required to remediate a certain concentration of nutrient.*

k	t_1	e^{kt1}	$e^{kt1} - 1$	a_0
0.424	4	5.462	4.462	6.626

a_0	$N_Ak/a_0 + 1$	$\ln(N_Ak/a_0 + 1)$	t_1
2.000	15.78	2.76	6.50

C2: Growth rate comparison in Growth Medium

Table 32: Mean OD values (n=3) and mean growth rate (k) values in growth medium in Experiment 1 between days 1-16.

Experiment 1 – GrM		
Day	**OD**	**k (d^{-1})**
1	0.146	0.599
5	0.538	0.381
6	0.696	0.360
7	0.839	0.336
3	0.215	0.329
8	0.945	0.309
9	1.038	0.285
2	0.139	0.276
12	1.224	0.227
16	1.333	0.176
0	0.080	

The growth rate on day 1 is an anomaly, caused by flocculation affecting OD measurements (Table 32).

Table 33: Mean OD values (n=3) and mean growth rate (k) values in growth medium in Experiment 4 between days 1-35.

Experiment 4 - GrM-ad GrM		
Day	**OD**	**k (d^{-1})**
5	0.217	0.356
8	0.469	0.319
2	0.069	0.315
7	0.315	0.308
6	0.231	0.308
10	0.714	0.297
3	0.089	0.295
12	0.968	0.273
14	1.262	0.253
16	1.618	0.237
18	1.944	0.221
20	2.176	0.204
22	2.281	0.188
24	2.504	0.176
26	2.565	0.164
30	2.688	0.143
35	2.947	0.125
1	0.029	-0.230
0	0.037	

Table 34: Mean OD values (n=3) and mean growth rate (k) values in growth medium in Experiment 4 between days 1-14.

Experiment 8 - GrM-ad BBM-H_2O		
Day	**OD**	**k (d^{-1})**
6	0.471	0.445
5	0.281	0.430
2	0.076	0.422
8	0.815	0.402
4	0.154	0.388
10	0.986	0.341
11	1.050	0.315
13	1.181	0.276
14	1.236	0.260
1	0.036	0.097
0	0.033	

The growth rate (k) was calculated between the start of the experiment and the day that an OD measurement was taken. So the k value on day 1 describes the growth rate between day 0 and day 1. The k value of day 6 is the growth rate between day 0 and day 6. Only microalgae previously cultured in growth medium was used to avoid confusion.

Appendix D: Standard Errors

D1: Initial Data

Table 35 and Table 36 show the standard errors of the IC data presented in Figure 7, Figure 8 and Figure 9.

Table 35: Standard errors of AD1, AD2901, AD1703 and BBM anion IC measurements. N/A = only one sample taken; n.a. = ion concentrations too low to measure in n or n-1 of the samples.

Sample	Month Sampled	n	Fluoride (as F)	Chloride (as Cl)	Nitrite (as N)	Bromide (as Br)	Nitrate (as N)	Sulphate (as S)	Phosphate (as P)	Nitrite (as N)
AD1	November	1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
AD2901	January	5	0.723	6.875	n.a.	n.a.	n.a.	3.118	6.444	n.a.
AD1703	March	3	0.145	21.544	n.a.	n.a.	n.a.	3.061	5.402	n.a.
BBM	April	1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table 36: Standard errors of AD1, AD2901, AD1703 and BBM cation IC measurements. N/A = only one sample taken; n.a. = ion concentrations too low to measure in n or n-1 of the samples.

Sample	Month Sampled	n	Sodium	Ammonium (as NH_4^+)	Potassium	Magnesium	Calcium
AD1	November	1	N/A	N/A	N/A	N/A	N/A
AD2901	January	5	33.980	98.944	10.333	1.023	1.364
AD1703	March	3	19.200	69.914	3.186	0.608	1.153
BBM	April	1	N/A	N/A	N/A	N/A	N/A

Table 37: Mean, standard deviations and relative standard deviations of AD1, AD2901 and AD1703 IC data

	Fluoride (as F)	Nitrite (as N)	Bromide (as Br)	Nitrate (as N)	Sulphate (as S)	Magnesium	Calcium	Phosphate (as P)	Chloride (as Cl)	Sodium	Potassium	Ammonium (as NH_4^+)
Mean:	10.52	0.12	1.84		14.46	1.38	9.47	103.78	203.04	156.77	120.54	1186.97
Std:	3.13	0.00	0.00		2.86	0.46	1.13	12.36	14.03	14.87	17.19	116.41
RStd:	30%	0%	0%		20%	33%	12%	12%	7%	9%	14%	10%

D2: Experiment 1

Table 38 shows the standard errors of the OD data presented in Figure 10, Experiment 1.

Table 38: Standard errors of OD measurements taken in Experiment 1

Hour	GM	0%	1%	10%	40%	70%	100%	100% - M
25.5	0.018	0.033	0.006	0.006	0.004	0.008	0.028	0.019
45.5	0.011	0.040	0.015	0.002	0.004	0.007	0.007	0.013
70.0	0.011	0.059	0.019	0.003	0.006	0.010	0.001	0.031
96.5	0.014	0.058	0.015	0.014	0.004	0.015	0.013	0.015
142.5	0.011	0.064	0.012	0.010	0.075	0.008	0.001	0.012
166.5	0.009	0.062	0.033	0.014	0.090	0.008	0.009	0.021
190.5	0.050	0.069	0.009	0.011	0.086	0.010	0.012	0.013
212.5	0.053	0.069	0.037	0.009	0.119	0.010	0.003	0.010
237.0	0.072	0.067	0.033	0.007	0.128	0.014	0.008	0.009
308.0	0.051	0.087	0.043	0.082	0.124	0.011	0.009	0.013
404.0	0.064	0.081	0.034	0.320	0.159	0.009	0.137	0.010

D3: Experiment 2

Table 39 shows the standard errors of the OD data presented in Figure 11, Experiment 2.

Table 39: Standard errors of OD measurements taken in Experiment 2.

Standard Error – Experiment 2				
Hour	0	100	124	146.25
10%	0.001	0.023	0.027	0.026

D4: Experiment 3

The standard errors of the OD data measured in Experiment 3 are displayed in Table 40.

Table 40: Standard errors of OD measurements taken in Experiment 3.

Day:	1	2	3	4	5	6	8	9	10	16
Hour	0.0	25.0	48.3	73.0	97.0	122.3	169.0	193.5	218.0	363.3
10%	0.015	0.013	0.019	0.006	0.010	0.015	0.022	0.010	0.018	0.021
20%	0.005	0.001	0.004	0.014	0.018	0.033	0.026	0.042	0.029	0.038

Table 41 shows the standard errors of the pH data measured in Experiment 3.

Table 41: Standard errors of pH measurements taken in Experiment 3.

Day:	5	6	8	9	16
Date:	**11/04/2014**	**12/04/2014**	**14/04/2014**	**15/04/2014**	**22/04/2014**
Hour:	97.0	122.3	169.0	193.5	363.3
10%	0.083	0.083	0.083	0.000	0.144
20%	0.083	0.083	0.083	0.083	0.167

D5: Experiment 4

Table 42 shows the standard errors of the OD data presented in Figure 14, Experiment 4.

Table 42a & b: Standard errors of OD measurements taken in Experiment 4.

Date:	23/04/2014	24/04/2014	25/04/2014	26/04/2014	28/04/2014	29/04/2014	30/04/2014	01/05/2014	03/05/2014	05/05/2014
Hour:	0.00	22.50	47.25	72.50	119.00	142.75	168.00	190.75	237.25	287.5
GrM-ad GrM	0.007	0.006	0.015	0.025	0.058	0.068	0.088	0.130	0.117	0.113
GrM-ad 1%	0.002	0.001	0.001	0.012	0.032	0.021	0.047	0.044	0.029	0.023
GrM-ad 5%	0.002	0.002	0.002	0.005	0.004	0.013	0.011	0.008	0.013	0.015
GrM-ad 10%	0.001	0.001	0.001	0.002	0.003	0.002	0.001	0.002	0.001	0.001
10%-ad GrM	0.002	0.003	0.004	0.001	0.020	0.026	0.014	0.054	0.133	0.364
10%-ad 1%	0.003	0.003	0.001	0.007	0.019	0.033	0.031	0.034	0.037	0.050
10%-ad 5%	0.001	0.002	0.001	0.002	0.003	0.005	0.004	0.001	0.003	0.003
10%-ad 10%	0.002	0.002	0.000	0.002	0.002	0.001	0.002	0.003	0.004	0.005

07/05/2014	09/05/2014	11/05/2014	13/05/2014	15/05/2014	17/05/2014	19/05/2014	23/05/2014	28/05/2014
337	382.75	432.75	478.5	522.5	571.5	620.5	718.25	834.75
0.186	0.149	0.106	0.095	0.090	0.075	0.087	0.059	0.096
0.030	0.031	0.021	0.016	0.017	0.016	0.030	0.016	0.031
0.032	0.037	0.042	0.047	0.066	0.113	0.093	0.145	0.189
0.002	0.003	0.010	0.019	0.040	0.072	0.078	0.039	0.010
0.363	0.319	0.280	0.210	0.236	0.158	0.231	0.194	0.199
0.093	0.077	0.079	0.086	0.082	0.098	0.083	0.104	0.097
0.003	0.006	0.005	0.005	0.003	0.007	0.005	0.010	0.018
0.007	0.012	0.019	0.023	0.025	0.015	0.012	0.005	0.017

The standard errors of the pH data presented in Figure 16, Experiment 4 are shown in Table 43.

Table 43: Standard errors of pH measurements taken in Experiment 4.

Date	**23/04/2014**	**24/04/2014**	**28/04/2014**	**23/05/2014**	**28/05/2014**
Hour	**0.00**	**22.50**	**119.00**	**718.25**	**834.75**
GrM-ad GrM	0.00	0.00	0.14	0.28	0.03
GrM-ad 1 %	0.00	0.00	0.00	0.15	0.25
GrM-ad 5 %	0.00	0.00	0.00	0.05	0.49
GrM-ad 10 %	0.00	0.00	0.00	0.11	0.10
10%-ad GrM	0.00	0.00	0.00	0.32	0.06
10%-ad 1 %	0.08	0.00	0.00	0.09	0.43
10%-ad 5 %	0.00	0.00	0.00	0.02	0.02
10%-ad 10 %	0.00	0.00	0.00	0.03	0.04

Table 44 and Table 45 shows the standard errors of the IC data presented in Figure 17-Figure 24, Experiment 4.

Table 44: Standard errors of IC anion data measured in Experiment 4. All day 0 values are written as N/A as only one reading was taken. When there was insufficient data (i.e. an ion's concentration was too low to be recorded by the ion chromatographer) in two or more of the three samples, this was denoted as n.a.

Sample ID	Day	Amount (mg/l)						
		Fluoride (as F)	Chloride (as Cl)	Nitrite (as N)	Bromide (as Br)	Nitrate (as N)	Sulphate (as S)	Phosphate (as P)
GrM-ad GrM	Day 0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Day 6	0.2	4.5	0.01	n.a.	1.59	0.27	0.96
	Day 35	0.09	1.84	0	n.a.	n.a.	0.1	2.1
10%-ad GrM	Day 0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Day 6	0.06	1.3	0	n.a.	1.14	0.25	0.14
	Day 35	0.29	2.64	n.a.	n.a.	n.a.	0.29	4.64
GrM-ad 1%	Day 0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Day 6	0.05	0.74	0	n.a.	0.01	0.03	0.08
	Day 35	0.01	0.08	n.a.	n.a.	0	0	n.a.
10%-ad 1%	Day 0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Day 6	0.02	0.31	0	n.a.	0.01	0	0.02
	Day 35	0.05	0.18	0.02	n.a.	0.01	n.a.	0.05
GrM-ad 5%	Day 0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Day 6	0.54	5.92	0.01	n.a.	0.03	0.09	0.05
	Day 35	0.12	0.14	0.03	n.a.	0	0.01	0.86
10%-ad 5%	Day 0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Day 6	0.01	0.18	0	n.a.	0	0.09	0.1
	Day 35	0.01	0.47	0.02	n.a.	0	0	0.07
GrM-ad 10%	Day 0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Day 6	0.02	5.49	0.01	n.a.	0.01	0.02	0.29
	Day 35	0.09	1.92	0.02	0	0.01	0.07	0.07
10%-ad 10%	Day 0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Day 6	0	0.16	0	n.a.	0.01	0.01	0.05
	Day 35	0.04	0.58	0.01	n.a.	0	0.09	0.29

Table 45: Standard errors of IC cation data measured in Experiment 4. All day 0 values are written as N/A as only one reading was taken. When there was insufficient data (i.e. an ion's concentration was too low to be recorded by the ion chromatographer) in two or more of the three samples, this was denoted as n.a.

Sample ID	Amount (mg/l)				
	Sodium	**Ammonium (as NH_4^+)**	**Potassium**	**Magnesium**	**Calcium**
GrM-ad GrM	N/A	N/A	N/A	N/A	N/A
	20.57	0.01	2.39	0.12	0.15
	3.76	n.a.	4.84	0.16	0.10
10%-ad GrM	N/A	N/A	N/A	N/A	N/A
	1.00	0.01	0.24	0.08	0.16
	18.38	n.a.	1.81	0.33	0.25
GrM-ad 1%	N/A	N/A	N/A	N/A	N/A
	3.19	0.85	0.14	0.01	0.02
	0.21	0.01	0.01	0.00	0.02
10%-ad 1%	N/A	N/A	N/A	N/A	N/A
	0.14	0.55	0.05	n.a.	0.01
	0.32	1.29	0.42	0.00	0.01
GrM-ad 5%	N/A	N/A	N/A	N/A	N/A
	4.96	4.60	0.31	0.03	0.15
	0.83	6.45	0.84	0.00	0.04
10%-ad 5%	N/A	N/A	N/A	N/A	N/A
	0.35	1.00	0.84	0.01	0.11
	0.55	0.66	0.25	0.00	0.03
GrM-ad 10%	N/A	N/A	N/A	N/A	N/A
	5.27	35.35	0.13	0.02	0.08
	2.42	1.53	0.68	0.03	0.05
10%-ad 10%	N/A	N/A	N/A	N/A	N/A
	0.13	1.14	0.08	0.01	0.05
	0.47	0.97	0.30	0.06	0.13

D6: Experiment 8

Table 46 shows the standard errors of the OD data presented in **Figure 28**, Experiment 8.

Table 46: Standard errors of OD measurements taken in Experiment 8

Date:	31/07/2014	01/08/2014	02/08/2014	04/08/2014	05/08/2014	06/08/2014	08/08/2014	10/08/2014	11/08/2014	13/08/2014	14/08/2014
Hour:	0	21.75	40.5	94.75	117.5	140	190.75	237.5	259.25	309	334
GrM-ad H_2O-AD	0.003	0.003	0.004	0.009	0.009	0.010	0.005	0.007	0.006	0.009	0.014
GrM-ad BBM-AD	0.015	0.016	0.023	0.078	0.095	0.119	0.129	0.128	0.115	0.116	0.109
GrM-ad BBM (-NP)-AD	0.005	0.008	0.011	0.036	0.038	0.042	0.095	0.095	0.092	0.088	0.084
GrM-ad BBM-H_2O	0.008	0.013	0.015	0.039	0.065	0.101	0.126	0.141	0.152	0.130	0.134

Table 47 shows the standard errors of the pH data presented in Figure 29, Experiment 8.

Table 47: Standard errors of pH measurements taken in Experiment 8

Date:	31/07/14	01/08/14	04/08/14	05/08/14	06/08/14	11/08/14	13/08/14	14/08/14
Hour:	0	21.75	94.75	117.5	140	259.25	309	334
GrM-ad H_2O-AD	0.007	0.006	0.012	0.010	0.013	0.015	0.015	0.012
GrM-ad BBM-AD	0.000	0.003	0.012	0.015	0.029	0.033	0.044	0.050
GrM-ad BBM (-NP)-AD	0.003	0.000	0.007	0.017	0.021	0.044	0.046	0.045
GrM-ad BBM-H_2O	0.000	0.009	0.006	0.012	0.023	0.039	0.030	0.020
10%-ad H_2O-AD	0.003	0.003	0.007	0.017	0.006			0.009
10%-ad BBM-AD	0.003	0.003	0.007	0.009	0.018			0.043
10%-ad BBM (-NP)-AD	0.003	0.003	0.015	0.010	0.009			0.032
10%-ad BBM-H_2O	0.003	0.006	0.000	0.003	0.012			0.000

The standard errors of the IC data presented in Figure 30-Figure 32, Experiment 8 are shown in Table 48, Table 49, Table 50 and Table 51.

Table 48: Standard errors of IC anion data measured on 31/07/2014 in Experiment 8. N/A = only one sample taken; n.a.= three samples measured, but ion concentration was too low to measure in 2-3 of the samples; - = coeluting peaks mean quantification was not possible.

Sample ID	Amount (mg/l)						
	Fluoride (as F)	Chloride (as Cl)	Nitrite (as N)	Bromide (as Br)	Nitrate (as N)	Sulphate (as S)	Phosphate (as P)
GrM-ad H_2O-AD	0.050	1.775	n.a.	0.009	0.003	0.015	0.306
10%-ad H_2O-AD	0.015	0.803	n.a.	0.010	0.003	0.019	0.197
GrM-ad BBM-AD	0.119	1.249	n.a.	0.009	1.121	0.251	0.606
10%-ad BBM-AD	0.015	1.159	n.a.	0.007	0.996	0.232	0.973
GrM-ad BBM(-NP)-AD	0.009	1.498	n.a.	0.015	0.003	0.656	0.330
10%-ad BBM(-NP)-AD	0.010	5.781	n.a.	0.003	0.007	0.496	0.214
GrM-ad BBM	0.003	0.942	n.a.	n.a.	1.038	0.300	1.171
10%-ad BBM	0.012	1.861	n.a.	n.a.	0.617	0.178	0.504
Neat AD	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table 49: Standard errors of IC cation data measured on 31/07/2014 in Experiment 8. N/A = only one sample taken; n.a.= three samples measured, but ion concentration was too low to measure in 2-3 of the samples; - = coeluting peaks mean quantification was not possible.

Sample ID	Amount (mg/l)				
	Sodium	Ammonium (as NH_4^+)	Potassium	Magnesium	Calcium
GrM-ad H_2O-AD	3.044	12.955	0.489	0.046	0.071
10%-ad H_2O-AD	2.281	1.975	0.500	0.017	0.078
GrM-ad BBM-AD	2.397	4.714	1.475	0.129	0.144
10%-ad BBM-AD	1.518	3.023	1.025	0.279	0.319
GrM-ad BBM(-NP)-AD	0.476	1.535	0.331	0.278	0.329
10%-ad BBM(-NP)-AD	2.451	1.814	0.514	0.138	0.166
GrM-ad BBM	1.616	0.057	3.638	0.101	0.073
10%-ad BBM	6.891	0.055	1.088	0.124	0.104
Neat AD	N/A	N/A	N/A	N/A	N/A

Table 50: Standard errors of IC anion data measured on 14/08/2014 in Experiment 8. N/A = only one sample taken; n.a.= three samples measured, but ion concentration was too low to measure in 2-3 of the samples; - = coeluting peaks mean quantification was not possible.

Sample ID	Amount (mg/l)						
	Fluoride (as F)	Chloride (as Cl)	Nitrite (as N)	Bromide (as Br)	Nitrate (as N)	Sulphate (as S)	Phosphate (as P)
GrM-ad H_2O-AD	0.068	0.517	0.002	0.002	0.007	-	0.167
10%-ad H_2O-AD	0.249	0.236	0.001	0.009	0.005	0.021	0.231
GrM-ad BBM-AD	0.102	0.842	0.001	0.000	0.781	0.292	2.137
10%-ad BBM-AD	0.077	0.709	0.005	0.002	0.651	0.297	0.165
GrM-ad BBM(-NP)-AD	0.051	4.692	n.a.	0.011	0.003	0.207	1.076
10%-ad BBM(-NP)-AD	0.118	2.196	n.a.	0.012	0.005	0.250	0.383
GrM-ad BBM	0.061	0.813	n.a.	n.a.	-	0.209	1.025
10%-ad BBM	0.232	1.749	n.a.	n.a.	0.003	0.232	0.815

Table 51: Standard errors of IC cation data measured on 14/08/2014 in Experiment 8. N/A = only one sample taken; n.a.= three samples measured, but ion concentration was too low to measure in 2-3 of the samples; - = coeluting peaks mean quantification was not possible.

Sample ID	Amount (mg/l)				
	Sodium	Ammonium (as NH_4^+)	Potassium	Magnesium	Calcium
GrM-ad H_2O-AD	0.591	30.259	0.211	0.035	0.501
10%-ad H_2O-AD	1.707	2.758	0.387	0.007	0.528
GrM-ad BBM-AD	2.312	9.509	3.327	0.231	0.056
10%-ad BBM-AD	3.610	6.651	0.868	0.914	1.560
GrM-ad BBM(-NP)-AD	3.941	7.883	0.700	0.192	0.085
10%-ad BBM(-NP)-AD	0.332	6.631	1.773	0.092	0.063
GrM-ad BBM	4.507	0.336	0.489	0.342	0.212
10%-ad BBM	3.684	0.076	2.709	0.139	0.174

Appendix E: Nutrient Fluctuations

E1: Nitrogen and phosphorus values in Experiment 8

Table 52: N and P concentrations at the beginning and end of Experiment 8 (mg/l).

	31/07/2014					14/08/2014				
	Phosphate (as P)	Nitrate (as N)	Ammonium (as NH_4^+)	Ammonium (as NH_4^+-N)	Total N	Phosphate (as P)	Nitrate (as N)	Ammonium (as NH_4^+)	Ammonium (as NH_4^+-N)	Total N
A GrM-ad H2O-AD	11.91	0.08	148.40	115.23	115.30	6.01	0.05	152.04	118.06	118.10
B GrM-ad H2O-AD	11.50	0.06	180.76	140.35	140.41	6.57	0.06	155.53	120.76	120.83
C GrM-ad H2O-AD	10.86	0.06	191.51	148.70	148.76	6.42	0.09	63.06	48.96	49.05
A10%-adH2O-AD	10.85	0.07	183.89	142.78	142.85	10.20	0.07	164.51	127.74	127.80
B10%-adH2O-AD	11.07	0.05	181.79	141.16	141.21	9.57	0.06	156.19	121.28	121.34
C10%-adH2O-AD	11.52	0.05	188.48	146.35	146.40	9.45	0.07	164.42	127.67	127.74
AGrM-adBBM-AD	55.55	35.46	192.93	149.81	185.27	52.69	34.73	149.53	116.11	150.84
BGrM-adBBM-AD	55.30	35.23	179.90	139.69	174.92	45.32	36.70	121.13	94.06	130.75
CGrM-adBBM-AD	57.23	37.91	194.94	151.37	189.27	48.42	38.71	120.88	93.86	132.57
A10%-adBBM-AD	57.49	33.94	181.29	140.76	174.70	49.66	36.97	116.05	90.11	127.08
B10%-adBBM-AD	56.52	34.74	184.85	143.53	178.27	49.52	36.55	135.98	105.58	142.13
C10%-adBBM-AD	54.21	37.36	191.60	148.77	186.13	50.07	38.74	136.03	105.62	144.36
AGrM-adBBM(-NP)-AD	10.71	0.14	188.67	146.49	146.64	4.85	0.12	134.66	104.56	104.69
BGrM-adBBM(-NP)-AD	11.72	0.19	193.25	150.06	150.24	5.54	0.18	141.74	110.06	110.23
CGrM-adBBM(-NP)-AD	11.68	0.14	193.30	150.09	150.24	2.02	0.08	115.36	89.57	89.66
A10%-adBBM(-NP)-AD	10.90	0.06	184.44	143.21	143.27	5.92	0.14	138.29	107.38	107.52
B10%-adBBM(-NP)-AD	11.64	0.15	190.72	148.09	148.24	4.64	0.14	117.16	90.97	91.11
C10%-adBBM(-NP)-AD	11.23	0.09	187.43	145.54	145.62	5.59	0.10	135.53	105.23	105.33
AGrM-adBBM	50.14	43.57	0.07	0.05	43.62	42.22	0.00	0.03	0.02	0.02
BGrM-adBBM	48.92	38.55	0.24	0.19	38.74	44.02	9.79	1.03	0.80	10.59
CGrM-adBBM	46.18	42.53	0.24	0.18	42.71	40.47	0.00	0.01	0.01	0.01
A10%-adBBM	50.69	42.37	0.08	0.06	42.43	40.21	0.05	0.22	0.17	0.22
B10%-adBBM	49.07	40.63	0.16	0.12	40.76	41.89	0.00	0.25	0.19	0.19
C10%-adBBM	49.32	39.85	0.27	0.21	40.06	39.09	0.04	0.01	0.01	0.04
NeatBAD	108.12	0.81	1747.30	1356.75	1357.55					

Table 53: N and P consumption, N:P change and N:P consumption ratio in Experiment 8.

	Solution N:P				Consumption			Consumption %	
	31/07/2014	14/08/2014	Change		N consumed	P consumed	N:P Consumption Ratio	N consumed	P consumed
A GrM-ad H2O-AD	9.68	19.65	9.96		-2.80	5.90	**-0.5**	-2%	50%
B GrM-ad H2O-AD	12.21	18.39	6.18		19.59	4.93	**4.0**	14%	43%
C GrM-ad H2O-AD	13.69	7.64	-6.05		99.72	4.45	**22.4**	67%	41%
A 10%-ad H2O-AD	13.17	12.54	-0.63		15.05	0.65	**23.0**	11%	6%
B 10%-ad H2O-AD	12.76	12.68	-0.08		19.87	1.50	**13.2**	14%	14%
C 10%-ad H2O-AD	12.70	13.51	0.81		18.66	2.07	**9.0**	13%	18%
A GrM-ad BBM-AD	3.34	2.86	-0.47		34.43	2.86	**12.1**	19%	5%
B GrM-ad BBM-AD	3.16	2.89	-0.28		44.17	9.98	**4.4**	25%	18%
C GrM-ad BBM-AD	3.31	2.74	-0.57		56.70	8.82	**6.4**	30%	15%
A 10%-ad BBM-AD	3.04	2.56	-0.48		47.62	7.83	**6.1**	27%	14%
B 10%-ad BBM-AD	3.15	2.87	-0.28		36.14	7.00	**5.2**	20%	12%
C 10%-ad BBM-AD	3.43	2.88	-0.55		41.77	4.14	**10.1**	22%	8%
A GrM-ad BBM(-NP)-AD	13.69	21.57	7.89		41.95	5.86	**7.2**	29%	55%
B GrM-ad BBM(-NP)-AD	12.82	19.91	7.09		40.01	6.18	**6.5**	27%	53%
C GrM-ad BBM(-NP)-AD	12.86	44.35	31.49		60.58	9.66	**6.3**	40%	83%
A 10%-ad BBM(-NP)-AD	13.15	18.17	5.02		35.75	4.98	**7.2**	25%	46%
B 10%-ad BBM(-NP)-AD	12.74	19.63	6.89		57.13	7.00	**8.2**	39%	60%
C 10%-ad BBM(-NP)-AD	12.97	18.84	5.87		40.29	5.64	**7.1**	28%	50%
A GrM-ad BBM	0.87	0.00	-0.87		43.60	7.93	**5.5**	100%	16%
B GrM-ad BBM	0.79	0.24	-0.55		28.15	4.90	**5.7**	73%	10%
C GrM-ad BBM	0.92	0.00	-0.92		42.70	5.71	**7.5**	100%	12%
A 10%-ad BBM	0.84	0.01	-0.83		42.21	10.48	**4.0**	99%	21%
B 10%-ad BBM	0.83	0.00	-0.83		40.56	7.18	**5.6**	100%	15%
C 10%-ad BBM	0.81	0.00	-0.81		40.02	10.24	**3.9**	100%	21%
Neat AD	12.56								

Table 54: Mean N and P consumption in Experiment 8

	Mean Consumption				
	N consumed	**P consumed**	**N:P Consumption Ratio**	**N %**	**P %**
GrM-ad H_2O-AD	38.84	5.09	8.64	26%	44%
10%-ad H_2O-AD	17.46	1.08	18.12	12%	13%
GrM-ad BBM-AD	45.10	7.22	7.64	25%	13%
10%-ad BBM-AD	41.84	6.32	7.11	23%	11%
GrM-ad BBM(-NP)-AD	47.51	7.23	6.63	32%	63%
10%-ad BBM(-NP)-AD	44.39	5.87	7.50	30%	52%
GrM-ad BBM	38.15	6.18	6.24	91%	13%
10%-ad BBM	40.93	9.30	4.53	100%	19%

E2: Change in nutrient concentration vs. variance within the data in Experiment 8

Table 55: Mean and standard errors of ion concentration data collected in Experiment 8

Means

Day 0	Amount (mg/l)												
	Fluoride (as F)	Chloride (as Cl)	Nitrite (as N)	Bromide (as Br)	Nitrate (as N)	Sulphate (as S)	Phosphate (as P)	Sodium	Ammonium (as NH_4^+)	Potassium	Magnesium	Calcium
GrM-add H2O-AD	0.55	27.06	0.00	1.05	0.75	1.90	11.42	91.47	173.55	19.06	0.14	0.72
10%-add H2O-AD	0.46	30.44	0.00	1.06	0.77	1.88	11.15	97.45	184.72	19.81	0.11	0.96
GrM-add BBM-AD	0.45	44.67	0.00	1.07	37.26	11.17	56.03	162.51	189.26	97.99	5.11	5.67
10%-add BBM-AD	0.56	43.31	0.00	1.06	36.08	10.94	56.07	165.25	185.91	96.30	5.06	5.48
GrM-add BBM(-NP)-AD	0.56	207.45	0.00	1.09	0.77	11.09	11.37	171.41	191.74	99.96	5.42	5.84
10%-add BBM(-NP)-AD	0.56	206.30	0.00	1.10	0.77	10.98	11.26	169.36	187.53	97.78	5.53	5.99
GrM-add BBM	0.16	32.12	0.00	0.00	42.10	11.51	48.42	187.11	0.18	91.43	5.92	4.82
10%-add BBM	0.16	27.79	0.00	0.00	41.65	11.34	49.70	172.39	0.17	95.72	5.62	4.48

Day 14	Amount (mg/l)											
	Fluoride (as F)	Chloride (as Cl)	Nitrite (as N)	Bromide (as Br)	Nitrate (as N)	Sulphate (as S)	Phosphate (as P)	Sodium	Ammonium (as NH_4^+)	Potassium	Magnesium	Calcium
GrM-add H2O-AD	1.45	30.30	0.47	0.57	0.77	0.00	6.33	98.37	123.54	19.75	1.43	2.31
10%-add H2O-AD	1.21	29.53	0.47	0.58	0.77	1.25	9.74	94.60	161.71	19.31	1.37	2.31
GrM-add BBM-AD	1.55	42.47	0.47	0.59	38.00	7.71	48.81	165.29	130.51	90.20	2.61	5.72
10%-add BBM-AD	0.93	43.54	0.48	0.57	38.71	7.61	49.75	166.04	129.35	96.03	1.94	4.54
GrM-add BBM(-NP)-AD	1.51	216.76	0.00	0.59	0.81	8.33	4.14	173.83	130.59	99.73	3.47	6.65
10%-add BBM(-NP)-AD	1.00	219.62	0.45	0.60	0.79	8.20	5.38	174.34	130.33	95.48	3.85	6.88
GrM-add BBM	0.66	30.45	0.00	0.00	10.17	7.98	42.24	190.96	0.36	92.07	4.18	5.60
10%-add BBM	1.29	36.43	0.00	0.00	0.75	7.71	40.40	197.56	0.16	81.91	4.13	5.33

Standard errors

Day 0	Amount (mg/l)											
	Fluoride (as F)	Chloride (as Cl)	Nitrite (as N)	Bromide (as Br)	Nitrate (as N)	Sulphate (as S)	Phosphate (as P)	Sodium	Ammonium (as NH_4^+)	Potassium	Magnesium	Calcium
GrM-add H2O-AD	0.05	1.77	n.a.	0.01	0.00	0.02	0.31	3.04	12.96	0.49	0.05	0.07
10%-add H2O-AD	0.01	0.80	n.a.	0.01	0.00	0.02	0.20	2.28	1.98	0.50	0.02	0.08
GrM-add BBM-AD	0.12	1.25	n.a.	0.01	1.12	0.25	0.61	2.40	4.71	1.47	0.13	0.14
10%-add BBM-AD	0.01	1.16	n.a.	0.01	1.00	0.23	0.97	1.52	3.02	1.02	0.28	0.32
GrM-add BBM(-NP)-AD	0.01	1.50	n.a.	0.02	0.00	0.66	0.33	0.48	1.54	0.33	0.28	0.33
10%-add BBM(-NP)-AD	0.01	5.78	n.a.	0.00	0.01	0.50	0.21	2.45	1.81	0.51	0.14	0.17
GrM-add BBM	0.00	0.94	n.a.	n.a.	1.04	0.30	1.17	1.62	0.06	3.64	0.10	0.07
10%-add BBM	0.01	1.86	n.a.	n.a.	0.62	0.18	0.50	6.89	0.06	1.09	0.12	0.10

Day 14	Amount (mg/l)											
	Fluoride (as F)	Chloride (as Cl)	Nitrite (as N)	Bromide (as Br)	Nitrate (as N)	Sulphate (as S)	Phosphate (as P)	Sodium	Ammonium (as NH_4^+)	Potassium	Magnesium	Calcium
GrM-add H2O-AD	0.07	0.52	0.00	0.00	0.01	n.a.	0.17	0.59	30.26	0.21	0.04	0.50
10%-add H2O-AD	0.25	0.24	0.00	0.01	0.00	0.02	0.23	1.71	2.76	0.39	0.01	0.53
GrM-add BBM-AD	0.10	0.84	0.00	0.00	0.78	0.29	2.14	2.31	9.51	3.33	0.23	0.06
10%-add BBM-AD	0.08	0.71	0.00	0.00	0.65	0.30	0.17	3.61	6.65	0.87	0.91	1.56
GrM-add BBM(-NP)-AD	0.05	4.69	n.a.	0.01	0.00	0.21	1.08	3.94	7.88	0.70	0.19	0.09
10%-add BBM(-NP)-AD	0.12	2.20	n.a.	0.01	0.00	0.25	0.38	0.33	6.63	1.77	0.09	0.06
GrM-add BBM	0.06	0.81	n.a.	n.a.	n.a.	0.21	1.03	4.51	0.34	0.49	0.34	0.21
10%-add BBM	0.23	1.75	n.a.	n.a.	0.00	0.23	0.81	3.68	0.08	2.71	0.14	0.17

Table 56: Calculated differences between mean ion concentrations on day 0 and day 14 of Experiment 8.

Change in Means

Sample ID	Amount (mg/l)							Amount (mg/l)				
	Fluoride (as F)	Chloride (as Cl)	Nitrite (as N)	Bromide (as Br)	Nitrate (as N)	Sulphate (as S)	Phosphate (as P)	Sodium	Ammonium (as NH_4^+)	Potassium	Magnesium	Calcium
GrM-add H2O-AD	0.90	3.24	0.47	-0.48	0.01	-1.90	-5.09	6.90	-50.01	0.69	1.29	1.60
10%-add H2O-AD	0.74	-0.91	0.47	-0.49	0.00	-0.63	-1.41	-2.85	-23.01	-0.50	1.26	1.36
GrM-add BBM-AD	1.10	-2.20	0.47	-0.48	0.75	-3.45	-7.22	2.78	-58.74	-7.79	-2.51	0.06
10%-add BBM-AD	0.38	0.22	0.48	-0.48	2.64	-3.33	-6.32	0.79	-56.56	-0.27	-3.13	-0.94
GrM-add BBM(-NP)-AD	0.95	9.31	0.00	-0.50	0.04	-2.77	-7.23	2.42	-61.15	-0.23	-1.96	0.81
10%-add BBM(-NP)-AD	0.43	13.32	0.45	-0.50	0.03	-2.78	-5.87	4.98	-57.21	-2.30	-1.68	0.89
GrM-add BBM	0.51	-1.67	0.00	0.00	-31.92	-3.53	-6.18	3.84	0.18	0.64	-1.74	0.78
10%-add BBM	1.13	8.63	0.00	0.00	-40.89	-3.63	-9.30	25.18	-0.01	-13.81	-1.49	0.86

Increase
Decrease
No change

It is necessary to factor in the standard errors to determine whether the increase/decrease between days 0-14 is real, or simply variance within the data. For a change to be significant, the lower and upper limits of the data points (i.e. the mean ± the standard error) must not overlap.

Equation 1

$$Increase = (\mu_{14} - S.E._{14}) - (\mu_0 + S.E._0)$$

Equation 2

$$Decrease = (\mu_{14} + S.E._{14}) - (\mu_0 - S.E._0)$$

Table 57: Mean ion concentrations ± standard error on day 0 and day 14 of Experiment 8.

μ_{0} ± $S.E._{0}$

Day 0	Amount (mg/l)							Amount (mg/l)				
	Fluoride (as F)	Chloride (as Cl)	Nitrite (as N)	Bromide (as Br)	Nitrate (as N)	Sulphate (as S)	Phosphate (as P)	Sodium	Ammonium (as NH_4^+)	Potassium	Magnesium	Calcium
GrM-ad H2O-AD	0.50	25.28	n.a.	1.05	0.75	1.88	11.12	88.43	160.60	18.57	0.10	0.64
10%-ad H2O-AD	0.45	29.64	n.a.	1.05	0.76	1.86	10.95	95.17	182.74	19.31	0.09	0.88
GrM-ad BBM-AD	0.33	43.42	n.a.	1.06	36.14	10.91	55.42	160.11	184.54	96.52	4.98	5.52
10%-ad BBM-AD	0.54	42.15	n.a.	1.05	35.08	10.71	55.10	163.74	182.89	95.28	4.78	5.16
GrM-ad BBM(-NP)-AD	0.55	205.95	n.a.	1.07	0.76	10.44	11.04	170.94	190.20	99.63	5.14	5.51
10%-ad BBM(-NP)-AD	0.55	200.52	n.a.	1.09	0.76	10.48	11.04	166.91	185.72	97.26	5.39	5.83
GrM-ad BBM	0.15	31.18	n.a.	n.a.	41.06	11.21	47.25	185.50	0.12	87.80	5.82	4.74
10%-ad BBM	0.14	25.93	n.a.	n.a.	41.03	11.16	49.19	165.50	0.12	94.63	5.50	4.37

μ_{0} ± $S.E._{0}$

Day 0	Amount (mg/l)							Amount (mg/l)				
	Fluoride (as F)	Chloride (as Cl)	Nitrite (as N)	Bromide (as Br)	Nitrate (as N)	Sulphate (as S)	Phosphate (as P)	Sodium	Ammonium (as NH_4^+)	Potassium	Magnesium	Calcium
GrM-ad H2O-AD	0.60	28.83	n.a.	1.06	0.76	1.92	11.73	94.52	186.51	19.55	0.19	0.79
10%-ad H2O-AD	0.48	31.24	n.a.	1.07	0.77	1.90	11.34	99.74	186.69	20.31	0.13	1.03
GrM-ad BBM-AD	0.57	45.92	n.a.	1.08	38.38	11.42	56.63	164.90	193.97	99.47	5.24	5.81
10%-ad BBM-AD	0.57	44.47	n.a.	1.06	37.07	11.17	57.04	166.77	188.93	97.33	5.34	5.80
GrM-ad BBM(-NP)-AD	0.57	208.95	n.a.	1.10	0.77	11.75	11.70	171.89	193.27	100.29	5.70	6.17
10%-ad BBM(-NP)-AD	0.57	212.08	n.a.	1.10	0.77	11.47	11.47	171.81	189.34	98.29	5.67	6.16
GrM-ad BBM	0.16	33.06	n.a.	n.a.	43.14	11.81	49.59	188.73	0.24	95.07	6.02	4.89
10%-ad BBM	0.17	29.65	n.a.	n.a.	42.26	11.51	50.20	179.28	0.23	96.81	5.74	4.58

μ_{14} ± $S.E._{14}$

Day 14	Amount (mg/l)							Amount (mg/l)				
	Fluoride (as F)	Chloride (as Cl)	Nitrite (as N)	Bromide (as Br)	Nitrate (as N)	Sulphate (as S)	Phosphate (as P)	Sodium	Ammonium (as NH_4^+)	Potassium	Magnesium	Calcium
GrM-ad H2O-AD	1.38	29.78	0.47	0.57	0.76	n.a.	6.17	97.78	93.28	19.54	1.39	1.81
10%-ad H2O-AD	0.96	29.29	0.47	0.57	0.76	1.23	9.51	92.89	158.95	18.92	1.37	1.79
GrM-ad BBM-AD	1.45	41.63	0.46	0.59	37.22	7.42	46.67	162.98	121.00	86.88	2.38	5.67
10%-ad BBM-AD	0.86	42.83	0.47	0.57	38.06	7.31	49.58	162.43	122.70	95.16	1.02	2.98
GrM-ad BBM(-NP)-AD	1.46	212.07	n.a.	0.58	0.81	8.12	3.06	169.89	122.70	99.03	3.27	6.57
10%-ad BBM(-NP)-AD	0.88	217.42	n.a.	0.58	0.79	7.95	5.00	174.01	123.69	93.71	3.76	6.82
GrM-ad BBM	0.60	29.64	n.a.	n.a.	n.a.	7.77	41.21	186.45	0.02	91.58	3.84	5.39
10%-ad BBM	1.05	34.68	n.a.	n.a.	0.75	7.48	39.58	193.88	0.09	79.20	3.99	5.16

μ_{14} ± $S.E._{14}$

Day 14	Amount (mg/l)							Amount (mg/l)				
	Fluoride (as F)	Chloride (as Cl)	Nitrite (as N)	Bromide (as Br)	Nitrate (as N)	Sulphate (as S)	Phosphate (as P)	Sodium	Ammonium (as NH_4^+)	Potassium	Magnesium	Calcium
GrM-ad H2O-AD	1.52	30.81	0.47	0.57	0.77	n.a.	6.50	98.96	153.80	19.96	1.46	2.82
10%-ad H2O-AD	1.45	29.76	0.47	0.58	0.77	1.27	9.97	96.31	164.46	19.70	1.38	2.84
GrM-ad BBM-AD	1.65	43.31	0.47	0.59	38.78	8.01	50.95	167.60	140.02	93.53	2.84	5.78
10%-ad BBM-AD	1.01	44.25	0.48	0.57	39.36	7.90	49.91	169.65	136.00	96.90	2.85	6.10
GrM-ad BBM(-NP)-AD	1.56	221.45	n.a.	0.60	0.81	8.53	5.21	177.77	138.47	100.43	3.66	6.74
10%-ad BBM(-NP)-AD	1.11	221.81	n.a.	0.61	0.80	8.44	5.77	174.68	136.96	97.25	3.94	6.94
GrM-ad BBM	0.72	31.26	n.a.	n.a.	n.a.	8.19	43.26	195.46	0.69	92.56	4.53	5.81
10%-ad BBM	1.52	38.17	n.a.	n.a.	0.75	7.94	41.21	201.25	0.24	84.62	4.27	5.51

Table 58 is composite table. Cells in which the mean nutrient concentrations had increased (in Table 56) used Equation 1 to determine whether the standard errors of the two data points overlapped, and cells in which the mean had decreased used Equation 2. If the cells that were positive in Table 56 remained positive, they were left as purple. Similarly if the values remained negative after being subjected to equation 2, the cell remained green. However if the positive values became negative, or vice versa, this indicated that the standard errors overlapped and the apparent change in nutrient concentrations may simply be variance within the data. Thus these cells were also changed to orange.

Table 58: Nutrient change in cultures in Experiment 8. Purple = significant increase; green = significant decrease; orange = insignificant change.

	Amount (mg/l)							Amount (mg/l)				
	Fluoride (as F)	Chloride (as Cl)	Nitrite (as N)	Bromide (as Br)	Nitrate (as N)	Sulphate (as S)	Phosphate (as P)	Sodium	Ammonium (as NH_4^+)	Potassium	Magnesium	Calcium
GrM-ad H2O-AD	0.78	0.95	n.a.	-0.47	0.00	n.a.	-4.62	3.26	-6.80	-0.01	1.21	1.03
10%-ad H2O-AD	0.48	0.13	n.a.	-0.47	0.00	-0.59	-0.98	1.13	-18.28	0.39	1.24	0.75
GrM-ad BBM-AD	0.88	-0.11	n.a.	-0.47	-1.16	-2.91	-4.48	-1.92	-44.52	-2.99	-2.15	-0.14
10%-ad BBM-AD	0.29	-1.64	n.a.	-0.48	0.99	-2.80	-5.19	-4.34	-46.88	1.62	-1.93	0.94
GrM-ad BBM(-NP)-AD	0.89	3.12	0.00	-0.47	0.04	-1.90	-5.83	-2.00	-51.73	0.80	-1.49	0.39
10%-ad BBM(-NP)-AD	0.31	5.34	n.a.	-0.48	0.01	-2.04	-5.28	2.20	-48.76	-0.01	-1.45	0.66
GrM-ad BBM	0.44	0.09	0.00	0.00	n.a.	-3.02	-3.98	-2.28	-0.21	-3.49	-1.29	0.50
10%-ad BBM	0.89	5.02	0.00	0.00	-40.27	-3.22	-7.98	14.60	0.12	-10.01	-1.23	0.58

Appendix F: Growth rate figures

Experiment 1

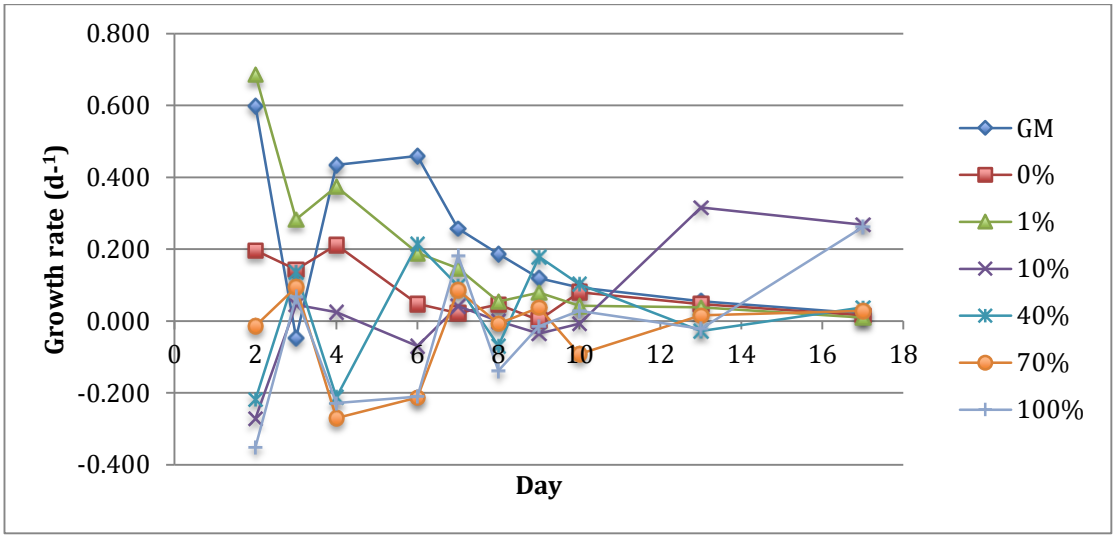

Figure 58: Growth rates of *Scenedesmus obliquus grown* in Bold's Basal Medium (GM) or water mixed with varying concentrations of ADL (0-100 % ADL). Growth rates were calculated using the equation $[\ln(Pt_1) - \ln(Pt_0)]/(t_1-t_0)$, with P referring to the mean optical density measurement on a given day (n = 3 flasks for each data series/culture media) and t referring to time in days.

Experiment 2

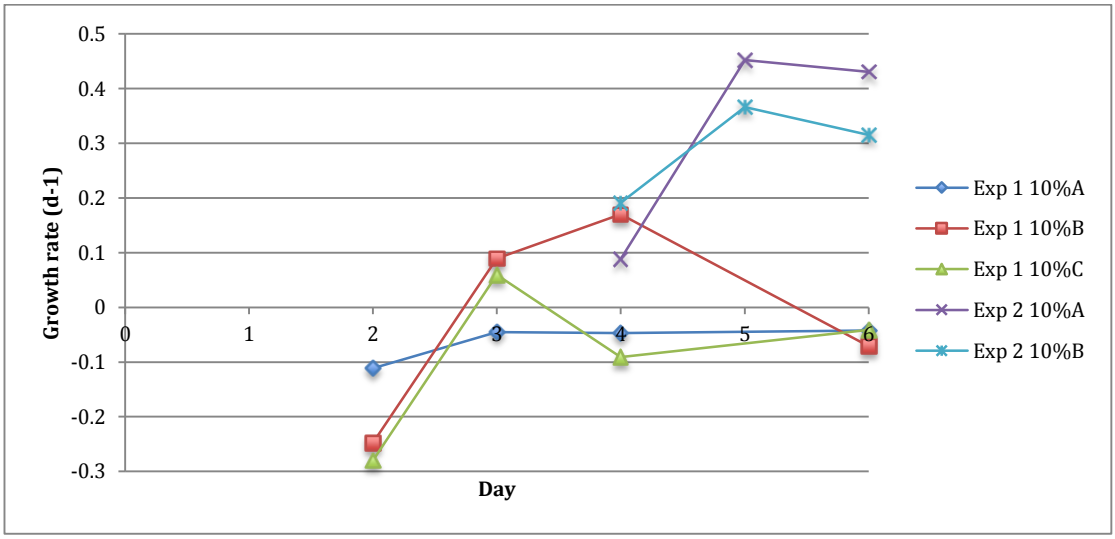

Figure 59: Growth rates of *Scenedesmus obliquus* cultures growing in five flasks on culture media containing 10 % ADL and 90 % deionized water; three flasks from Experiment 1 are shown (Exp 1 10 % A, B & C) and two flasks from Experiment 2 (Exp 2 10 % A & B). Growth rates were calculated using the equation $[\ln(Pt_1) - \ln(Pt_0)]/(t_1-t_0)$, with P referring to the optical density measurement on a given day and t referring to time in days.

Experiment 3

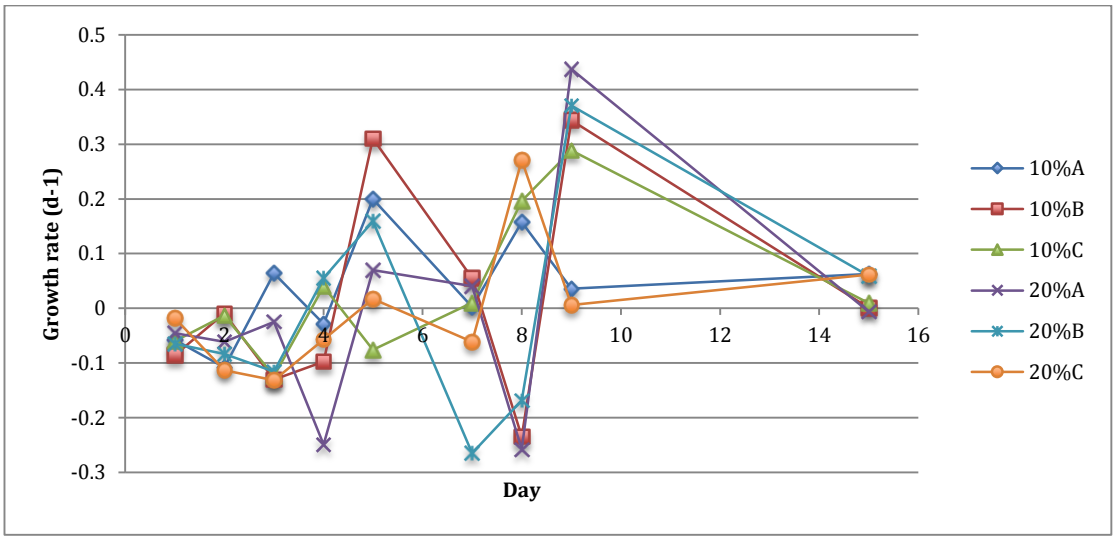

Figure 60: Growth rates of *Scenedesmus obliquus* cultures grown in six flasks on culture media made up of deionized water and 10 % ADL (10 % A, B and C) or 20 % ADL (20 % A, B & C). Growth rates were calculated using the equation $[\ln(Pt_1) - \ln(Pt_0)]/(t_1-t_0)$, with P referring to the optical density measurement on a given day and t referring to time in days.

Experiment 4

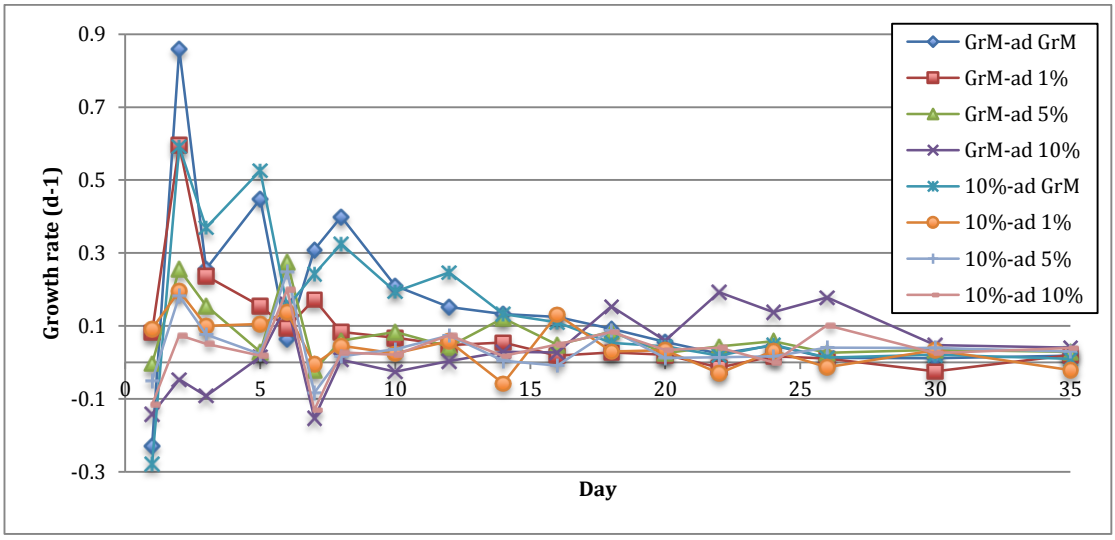

Figure 61: Growth rates of *Scenedesmus obliquus* grown in Bold's Basal Medium (GrM) or water mixed with varying concentrations of ADL (1-10 % ADL). *S. obliquus* was sub-cultured from either a flask in which the microalgae were growing on Bold's Basal Medium (GrM-ad) or a flask in which 10 % ADL was the culture medium (10%-ad). Growth rates were calculated using the equation $[\ln(Pt_1) - \ln(Pt_0)]/(t_1-t_0)$, with P referring to the mean optical density measurement on a given day (n = 3 flasks for each data series) and t referring to time in days.

Experiment 6

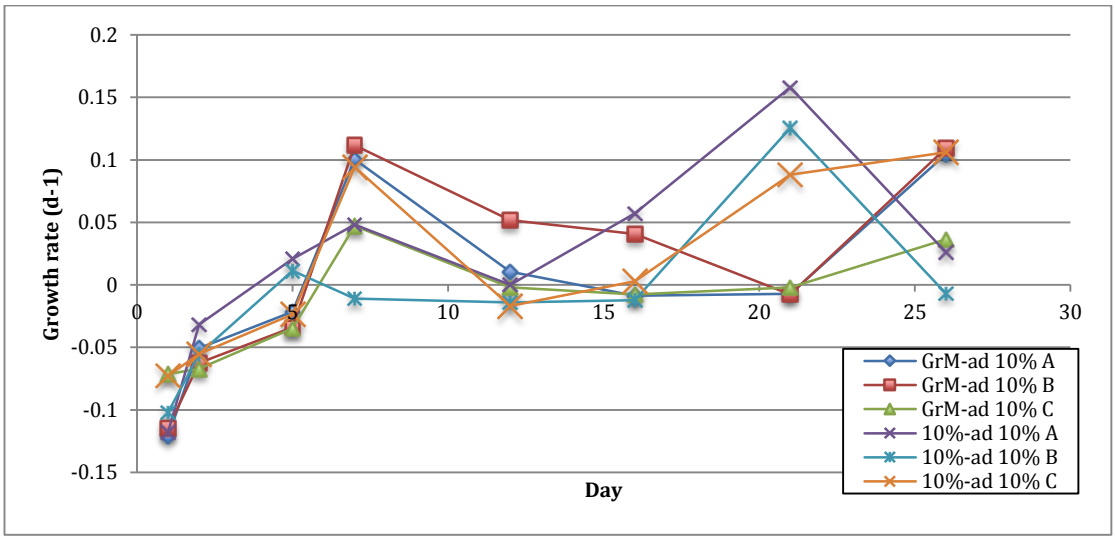

Figure 62: Growth rates of *Scenedesmus obliquus* cultures grown in six flasks on culture media made up of deionized water and 10 % ADL. *S. obliquus* was sub-cultured from either a flask in which the microalgae were growing on Bold's Basal Medium (GrM-ad) or a flask in which 10 % ADL was the culture medium (10%-ad). Growth rates were calculated using the equation $[\ln(Pt_1) - \ln(Pt_0)]/(t_1-t_0)$, with P referring to the optical density measurement on a given day and t referring to time in days.

Experiment 8

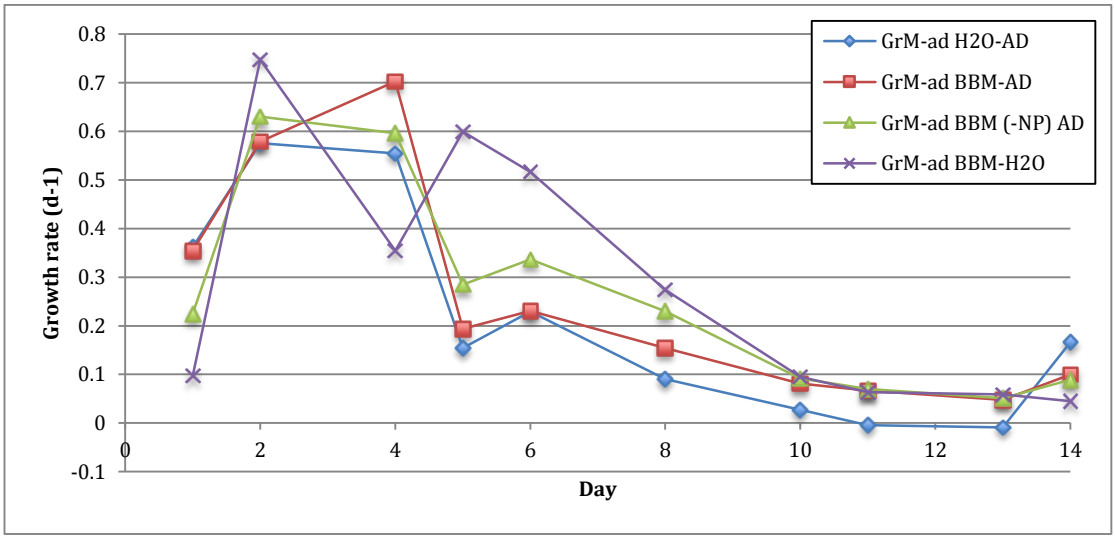

Figure 63: Growth rates of *Scenedesmus obliquus* grown on culture media containing: 10 % ADL and 90 % deionized water (H_2O-AD); 10 % ADL and 90 % Bold's Basal Medium (BBM-AD); 10 % ADL and 90 % modified Bold's Basal Medium containing no phosphorus or nitrogen (BBM (-NP) AD); and 10 % deionized water and 90 % Bold's Basal Medium. All cultures were sub-cultured from a flask in which *S. obliquus* was growing on Bold's Basal Medium (GrM-ad). Growth rates were calculated using the equation $[\ln(Pt_1) - \ln(Pt_0)]/(t_1-t_0)$, with P referring to the mean optical density measurement on a given day (n = 3 flasks for each data series) and t referring to time in days.

Appendix G: Anomalous sodium and chloride concentrations

In Experiment 4, anomalously high concentrations of sodium and chloride were recorded in the GrM-ad cultures growing in flasks containing 1 %, 5 % and 10 % ADL (Figure 19, 21, 23). This appendix outlines the arguments that show that this was not a sampling error but a real effect.

Table 59: Overview of the initial conditions of the 24 flasks used in Experiment 4. Each flask contained 30 ml of liquid (1 %, 5 % or 10 % ADL diluted with deionized water, or Bold's Basal Medium) and was inoculated with *Scenedesmus obliquus* previously grown in BBM (GrM-ad) or 10 % ADL (10%-ad). Each condition was repeated in triplicate (x3 flasks). The red cells show the flasks which contained anomalously high concentrations of Na and Cl measured on day 6 of the experiment.

	Microalgae pre-incubation environment	
Media	**BBM (GrM-ad)**	**10% ADL (10%-ad)**
BBM	x3 flasks	x3 flasks
1% ADL	x3 flasks	x3 flasks
5% ADL	x3 flasks	x3 flasks
10% ADL	x3 flasks	x3 flasks

Table 59 is has been modified from Table 4 in the Methods section and shows the flasks in which the anomalously high concentrations of sodium and chloride were measured. It shows that out of the 24 flasks measured in the study (three times on day 0, 6 and 35), only 9 flasks contained the anomalously high concentrations of sodium and chloride. These anomalously high concentrations were consistent across each replicate in these cultures (see Table 60), so were not caused by single anomalies but a consistent effect observed across 9 flasks.

Table 60: Relative standard deviations of chloride and ion concentrations in subset of flasks used in Experiment 4 in which anomalously high concentrations of sodium and chloride were measured on day 6. The flasks were inoculated with *Scenedesmus obliquus* that had previously been growing in Bold's Basal Medium (GrM-ad), growing on 1 %, 5 % and 10 % ADL diluted with deionized water.

Culture	**Chloride**	**Sodium**
GrM-ad 1%	2.25 %	8.59 %
GrM-ad 5%	15.6 %	12.6 %
GrM-ad 10%	11.9 %	11.2 %

As it appears that these high concentrations are not due to anomalous measurements in single flasks, the other issue that could have called into question the reliability of these results is sampling error. However that seems unlikely as the effect is observed in 9 flasks, but not in the other 9 flasks containing ADL dilutions (10%-ad 1%, 10%-ad 5% and 10%-ad 10%) or the other 3 flasks containing *S. obliquus* previously grown on BBM (GrM-ad GrM). All of these flasks were sampled on the same day, stored in the same conditions for the same duration of time, and measured together in the same machine on the same runs. So it seems very unlikely that a sampling error would occur in 9 flasks

and not in the other 15 flasks. Additionally, as previously mentioned in Section 3.5.3.2, the sodium and chloride concentrations were measured on separate machine runs as cations and anions were measured separately. All flasks that contained a high sodium concentration also contained a high chloride concentration, not a single flask contained only one anomalously high concentration of sodium or chloride. So the anomaly actually appeared 18 times out of 48 runs. And though it could be argued that the sodium peak may be due to the fact that the sample was not measured on an optimal dilution (an estimate was made from the first neat run) the chloride concentrations were measured on an optimal dilution so it could not explain away both of these anomalies.

When all of these factors are considered it becomes apparent that the likelihood of these anomalies being due to sampling error is very low and it can only be concluded that this is in fact a real effect. Unfortunately due to time constraints it was not possible to determine the cause of this effect in the course of this project. However if the project were to be developed further it would be beneficial to further investigate what drives changes in sodium and chloride concentrations in ADL dilutions during growth experiments.

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